# THE CYTOTOXIC ACTIVITY OF NEWLY ISOLATED PHENOLIC COMPOUND FROM *PANDANUS ODORATTISSIMUS* AGAINST CANCER (*IN VIVO AND IN VITRO*)

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# DECLARATION

I hereby declare that the Post- Doctoral Research Report entitled **"THE CYTOTOXIC ACTIVITY OF NEWLY ISOLATED PHENOLIC COMPOUND FROM *PANDANUS ODORATTISSIMUS* Linn AGAINST CANCER (*IN VIVO AND IN VITRO*)"** is the outcome of the original Post-Doctoral Research work carried out by me, under the guidance of **DR. PARAMJYOTHI L SWAMY, Professor, Department of Biochemistry, Gulbarga University, Kalaburagi, Karnataka.** The research report or any part thereof has not been submitted for the award of any degree or diploma in any University or Institution.

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# CERTIFICATE

This is to certify that the Post-Doctoral Research Report entitled "**THE CYTOTOXIC ACTIVITY OF NEWLY ISOLATED PHENOLIC COMPOUND FROM *PANDANUS ODORATTISSIMUS* AGAINST CANCER (*IN VIVO* AND *IN VITRO*)"** submitted by **DR. ABHAYKUMAR KAMBLE**, for the award of **UGC-POST DOCTORAL FELLOWSHIP FOR SC/ST** in **BIOCHEMISTRY** embodies the research carried out by him under my supervision in the Department of Studies and Research in Biochemistry, Gulbarga University, Kalaburagi, Karnataka. The research report or any part thereof has not been previously submitted to any university for any degree or diploma.

Place: Kalaburagi

Date: 13-08-2020

**Dr. Paramjyothi L Swamy**

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**The Cytotoxic Activity of Newly Isolated Phenolic Compound from *Pandanus odorattissimus* Linn against Cancer (*Invivo* and *Invitro*)"**

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# ABBREVIATION

| | |
|---|---|
| EAC | Ehrlich Ascites Carcinoma |
| DAL | Dalton's Ascitic Lymphoma |
| HL-60 | Human leukemia |
| KB | KERATIN-forming tumor cell line |
| MCF-7 | Michigan Cancer Foundation-7 |
| MDA-MB-468 | Breast cancer cells |
| L929 | Noncancerous cell line |
| APL | Acute promyclocytic leukemia |
| MTT | 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide |
| LDH | Lactate Dehydrogenase |
| ER | Estrogen-receptor |
| TNF | Tumor Necrosis Factor |
| IL | Interleukins |
| IF | Interferons |
| HepG2 | Human liver carcinoma |
| DMSO | Dimethyl sulfoxide |
| DMEM | Dulbecco’s Modified Eagle's Medium |
| FBS | Fetal bovine serum |
| EMS | Ethyl methane suphonate |
| NMA | Normal Melting Agarose |
| LMPA | Low Melting Point Agarose |
| EtBr | Ethidium Bromide |
| PBS | Phosphate Buffered Saline |

# REVIEW OF LITERATURE

Cancer generates massive public burdens across the world, and the struggle to fight cancer and eventually find a cure is one of humankind's greatest battles. Drugs to fight cancer, and many other diseases, are discovered and developed from many different sources, which include natural products like plants. There are many drugs today that are used to fight cancer that were originally obtained from natural sources [1,2]. A large problem today is that conventional chemotherapy comes with severe side effects, such as chemical toxicity to the host cells. Thus, research is underway to study possible chemotherapy drugs that are derived from plants [3].

The medicinal plants have very important role in the health of human being as well as animals. In India, Ayurveda has integrated the concept of interconnectedness into its understanding of health and disease. The growing interest in Ayurveda is not only because it is free of synthetic chemicals, but also because of its integrated approach.

*Borassus flabellifer* showed good cytotoxic effect on EAC (Ehrlich Ascites Carcinoma) cell line and DAL (Dalton's Ascitic Lymphoma) cell line in both MTT assay and Trypan blue dye exclusion assay. The plant showed significant reduction in the percent increase in body weight, tumor volume, tumour weight, viable cell count and increased non- viable cell count. These results suggested that *Borassus flabellifer* exhibit significant anticancer activity towards both methods Studied by G. Babu and Jeenachandran[4].

Takaidza*et al.*,[5]studied the inhibitory effect of acetone and water extracts of *Tulbaghia violacea* on the growth of human oral cancer cells was observed to be dose dependent with $IC_{50}$ values of 0.2 mg/mL and 1 mg/mL, respectively. Cell death of KB cell lines (KERATIN-forming tumor cell line) suggests the potential of the crude extracts of *Tulbaghia violacea* on progression of cancer. Cell death can occur via apoptosis or necrosis.

The anticancer activity of the extracts of *Tulbaghia violacea* can be attributed to some secondary metabolites such as phenolic compounds which have been shown to inhibit the formation of tumours by interfering with the growth, proliferation, metastasis as well as pro-apoptotic effects by blocking proteasome activity and suppressing angiogenesis.

According to Teiten*et al.,* [6] allicin metabolites and diallylpolysulfides found in *Allium sativum* have been reported to be strong activators of early mitotic arrest, aberrant tubulin depolymerisation that prevents the synthesis of normal spindle microtubules and interruption of microtubular dynamics, therefore leading to G2/M arrest. The genetic relationship between *Allium sativum* and *Tulbaghia violacea* species may suggest that similar mechanisms of cell disturbances may be present in the species under consideration in this study.

The results are comparable to that of work done by Chung *et al.,* [7] where the plant extracts from Saudi Arabia possessed greater toxicity to human breast adenocarcinoma (MCF-7) cancer cell lines but did not confer any lethal effect on normal cell line.

A few studies have shown that *in-vitro* migration assay has been as a tool to measure the effect of plant extract on arresting cancer cell motility, indicating a crucial linkage between the cytotoxic compounds and metastasis.

Damodaran *et al.*,[8]focused on evaluating the cytotoxic, antiproliferative, and proapoptotic effects of *Calotropis gigantean* leaf extracts on cancer cell lines. They demonstrated that *Calotropis gigantean* leaf extracts are cytotoxic against HeLa, MCF7, and A549 cell lines. Among these cell lines, the extract exhibited the highest cytotoxicity against the lung cancer cell line, A549 ($IC_{50}$ = 27.32 μg/mL), followed by MCF7 ($IC_{50}$ = 43.65 μg/mL) and HeLa ($IC_{50}$ = 117.92 μg/mL) cell lines.

Gawlik-Dziki *et al.,* [9] studies reveal of *Chenopodium quinoa* Leaves (ChL) were assessed through analyses of their phenolic content, elucidation of the effect of ChL phenolic compounds on cancer cell properties and estimation of their antioxidative activity, bioaccessibility and bioavailability *in vitro*. Considerable amounts of ferulic, sinapinic and gallic acids, kaempferol, isorhamnetin and rutin were observed in the chemical chloroform extract and were linked with its inhibitory effect on prostate cancer cell proliferation, motility and cellular competence for gap junctional communication. Both extracts, chemical and obtained after simulated digestion, exerted an inhibitory effect on lipoxygenase activity, paralleled by their considerable chelating, antioxidative, antiradical and reducing power. These observations indicate that phenolic ChL compounds may exert a chemopreventive and anticarcinogenic effect on oxidative stress and ROS-dependent intracellular signaling via synergic effects. The relatively high potential

bioaccessibility and bioavailability of the compounds probably responsible for these effects demonstrates the suitability of ChL for dietary supplementation.

The cytotoxicity effect of tomato (*Lycopersicu mesculentum)* leaves (methanol extract) on cancer cells to address potential therapeutic in MCF-7 breast cancer cell lines and its toxicity towards Vero cells was shown. The effect of extract towards MCF-7 breast cancer cell lines and Vero cells were observed using *in vitro* cytotoxicity assay to indicate its active fractions and its half maximal inhibitory concentration ($IC_{50}$). Purified sample gave a rational effect towards MCF-7 breast cancer cells with $IC_{50}$ value of 5.85 μg/mL studied by WanChik *et al.*,[10].

Gerlach*et al*,.[11]studied that Cycloviolacin $O_2$ ($CyO_2$), a cyclotide from *Viola odorata* (Violaceae) has antitumor effects and causes cell death by membrane permeabilization. In the breast cancer line, MCF-7 and its drug resistant subline MCF7/ADR, the cytotoxic effects of CyO2 (0.2-10 microM) were monitored in the presence and absence of doxorubicin (0.1-5 microM) using cell proliferation assays to establish its chemosensitizing abilities. This study documents several cyclotides with robust cytotoxicity that may be promising chemosensitizing agents against drug resistant breast cancer.

Mahadik Vaishali *et al.,* [12] investigated the evaluation of antitumor and antioxidant activity of ethanolic extract of *Vitis vinifera* L. leaves. For its antitumor, antioxidant activity in Ehrlich ascites carcinoma (EAC) induced swiss albino mice were induced. The antitumor effect and antioxidant role was assessed using tumor volume, packed cell volume and estimation of liver LPO and antioxidant enzymes such as SOD and CAT. The ethanolic extract was

administered at 200 and 400 mg/kg b.w. per day for 14 days, after 24 hours of tumor inoculation. Treatment with extract at a dose of 200 and 400 mg/kg increased mean survival time. Treatment with extract also decreased the levels of LPO and increased the levels of superoxide dismutase and catalase. The results suggest that ethanolic extract of *Vitis vinifera* possess significant antitumor and antioxidant effects in EAC tumor bearing mice.

The antitumor efficacy of the drug formulas was shown in terms of % in change of body weight and MST. The variations in the body weight could be a sign of the drug toxicity that precede the animal death. The EAC control group has significantly increased in body weight than the other groups, which is attributed to the growth of the EAC in the peritoneal cavity studied by Raju *et al,*.[13]

These morphological alterations indicate the apoptosis of EAC cells which is the common way to expel the inappropriate and redundant cells from body through a series of processes without damaging any normal cells. Lacking of apoptosis process plays a vital role in tumor development that lead to abnormal cell proliferation as well as cancer development.

A recent study showed the detection of phenol,2,4-bis(1,1-dimethylethyl)- (1) with GC/MS in *Streptomyces cavouresis* KUV39 isolated from vermicompost samples in India and demonstrated that this compound exhibited potent antioxidant properties and cytotoxicity against Hela cells investigated by Narendhran et al.,[14].

It is a target for numerous biologically active phytochemicals, including curcumin, resveratrol and epigallocatechin gallate. Exposure of cells to stimuli such as PMA leads to IκBa phosphorylation and degradation, allowing NF-κB to translocate to the nucleus where it binds to the MMP-9 promotor and activates transcription.

Phenolic compounds, such as phenolic acids, flavonoids, quinones, coumarins, lignans, stilbenes and tannins, are rich in antioxidant properties. These antioxidant compounds possess anti-inflammatory, anti-atherosclerotic, anti-tumor, anti-mutagenic, anti-carcinogenic, anti-bacterial and anti-viral activities.

Kenny et al., [15] studied the plant extract that will act successfully as an anti-cancer drug should kill cancer cells without causing excessive damage to normal cells, such as L929. L929, MCF-7 and MCF-12A cells are p53 wild-types, while MDA-MB-468 and MDA-MB-231 cells are p53 mutations. Furthermore, MCF-7 cells are estrogen-receptor (ER) positive and classified as low-grade and luminal type. MDA-MB-468 and MDA-MB-231 cells are ER negative and classified as high-grade and basal type. Their results showed that had more cytotoxic effects on MCF-7, MCF-12A and MDA-MB-468 than MDA-MB-231, a high-grade cancer.

Kerr *et al.,* [16] investigated the apoptosis, the earliest recognized morphological changes are chromatin condensation and nuclear fragmentation. Progression of the condensation is

accompanied by convolution of the nuclear followed by breaking up of the nucleus into discrete fragments.

Mohammadi *et al.,* [17] studies strongly suggests that the dichloromethane extract of *Urtica dioica* may contain bioactive compound that kill human breast adenocarcinoma cells, MDA-MB-468 cells without inducing substantial damage to noncancerous cell line L929, thus possibly suggesting a new potential chemotherapeutic agent for the treatment of breast cancer.

*Sofi et al.,*[18] investigated the levels of cytosolic enzyme Lactate Dehydrogenase (LDH) which was significantly elevated after 48 h of *Abrus precatorius* leaf extract treatment in the medium when compared to the control. This is one of the important hallmarks of apoptosis is DNA fragmentation. As shown in agarose gel electrophoresis, increased DNA fragmentation was apparent in treated MDA-MB-231 and MCF-7 cells as compared to the untreated cells. Flow cytometric analysis showed that *Abrus precatorius*has a pro-apoptotic activity on both MDA-MB-231 and MCF-7 cells.

The water extract of *Opuntia ficus-indica* plant was screened for cytotoxic activities by standard MTT assay against three cancerous cell viz., A549, HeLa and MCF-7 and a normal HEK293 cell cultures. The successive water extract showed selective toxicity towards cancer cells. The successive aqueous fraction showed potent activity against HeLa cells with $IC_{50}$of 39.72µg/ml and 85.63µg/ml by MTT and SRB assay methods respectively and was

comparatively nontoxic towards normal HEK293 cell cultures. The aqueous extract had no activity against A549 and MCF-7 cell cultures.

The research was aimed to study the cytotoxic activity of methanolic extracts of *Artocarpus heterophyllus* plant by various *in vitro* cytotoxic assays like MTT and SRB against different cell lines like HEK293, A549, HeLa and MCF-7. The $IC_{50}$ values of methanolic extract of *Artocarpus heterophyllus* were found 35.26 μgm/ml and 35.27 μgm/ml against A549 cell line by MTT and SRB assay methods respectively whereas this extract was found to be non toxic to normal cells (HEK293), proved that the methanolic extract exhibited significant anti cancer potential with no toxicity on normal cell line.

Cytotoxic activity of *Astragalus chrysochlorus* crude extracts was investigated. Hexane, chloroform, ethyl acetate, 80% ethanol and water extracts prepared from roots and stems of *Astragalus chrysochlorus* were tested for cytotoxic activity on Vero (V) cells by the MTT reagent. MTT assay was used to investigate the reduction of viability of cell cultures in the presence and absence of the extracts.

The anticancer potential of methanol extract of *Mucuna pruriens* (L) DC. (seeds) against EAC bearing mice was studied and reported. The extract was administered at a dose level of 125 and 250mg/kg b.w. for 14 days, after 24 hours of tumor inoculation. Decrease in tumor volume, packed cell volume, and viable cell count were observed in MEMP treated animals as compared

to EAC treated animals. Treatment increased the mean survival time significantly. The plant extract showed a marked reversal of the Hematological parameters which included a significant increase in RBC count and decrease in WBC count on plant treated animals. The results clearly demonstrate the antitumor potential of the methanol extract of *Mucuna pruriens* seeds against EAC cell lines investigated by Y. Rajeshwar, Manali Gupta *et al.*, .[19]

The antitumor activity of ethanol extract of *Indigofera aspalathoides Vahl Ex* DC. (EIA) was evaluated against the Ehrlich ascites carcinoma (EAC) tumor model. The activity was assessed using survival time, peritoneal cell count, hematological studies, solid tumor mass and *in vitro* cytotoxicity. Oral administration of EIA increased the survival time and normal peritoneal cell count. The altered parameters on tumour bearing animals were restored on treatment with EIA. Short time *In-Vitro* cytotoxic studies also established the anticancer potential of the plant extract studied by Rajkapoor *et al.*,[20]

Amritpal Singh *et al.*,[21] studied the Withaferin A is a steroidal lactone, derived from *Acnistus arborescens*, showed marked tumor-inhibitory activity when tested *in-vitro* against cells derived from human carcinoma of nasopharynx (KB). It also acted as a mitotic poison arresting the division of cultured human larynx carcinoma cells at metaphase and in HeLa cultures similar to star-metaphase. It also produced significant retardation of the growth of Ehrlich ascites carcinoma, Sarcoma 180, Sarcoma Black (SBL), and E 0771 mammary adenocarcinoma in mice at the dose levels of 10, 12, 15 mg/kg body weight. Growth of Ehrlich ascites carcinoma was completely inhibited in more than half of the mice, which survived for 100 days without the

evidence of growth of the tumor. Withaferin-A caused mitotic arrest in embryonal chicken fibroblast cells.

Gayatri*et al.*,[22] investigated the crude extracts of *Piper longum* L. fruits, for short term *In vitro* and *In vivo* cytotoxic activity using Ehrlich ascites carcinoma cells. Swiss albino mice were used for the *In vivo* studies. Ehrlich ascites carcinoma cells were inoculated intraperitoneally. Petroleum ether and chloroform extracts had shown significant cytotoxic action on the EAC cell lines. Petroleum ether extract treated animals showed an increase in life span up to 44.44 %. But, chloroform extract increased the life span of tumor bearing animals only by 33.33%. An increase in life span up to 87.5% was observed in animals which were treated with both the extracts.

The anticancer effect of hydroalcoholic extract of *Aegle marmelos* (L.) Corr. (AME) was studied in the Ehrlich Ascites carcinoma bearing Swiss albino mice. The study was aimed to find out the $LD_{10}$ and $LD_{50}$ of the plant drug under study. The AME treatment resulted in a dose dependent elevation in the median survival time (MST) observed up to 400 mg/kg and declined thereafter. The acute toxicity study of AME showed that the drug was non-toxic up to a dose of 1750 mg/kg b. wt. The $LD_{10}$ and $LD_{50}$ were found to be 2000 and 2250 mg/kg respectively Ganesh Chandra Jagetia *et al.*,[23].

Senthilkumar Natesan *et al.*,[24] studied, the anticancer potentials of Methanol Extract of *Careya arborea Roxb*. (MEC) (bark) evaluated against Dalton's lymphoma ascites (DLA)-induced tumors. Methanol extract was administered orally to mice at the dose of 250 and 500 mg/kg body weight for 10 days. There was a significant reduction in body weight, viable tumor cell count and, packed cell volume as compared to the mice of control group. On administration of the drug, restoration of altered haematological and biochemical parameters were observed. Histological observations of liver and kidney also supported the anticancer activity of the plant extract.

Aqueous and ethanol extracts of *Youngia japonica* (L.) DC. (also known as Oriental hawksbeard) were tested *in vitro* for anti-tumor activity against three cell lines, human promyelocytic leukaemia (HL-60), human myelogenous leukaemia (chronic K562) and mouse Sarcoma 180 (S-180). Hot water extract of *Youngia japonica* inhibited cell proliferation and growth of all cancer cell lines to various extents. K-562 cells were most sensitive to the extract whereas S-180 cells were the least. It did not show any significant cytotoxic effects on normal mammalian Vero cells up to the concentration of 450 μg/ml.

Min-Jung Kim *et al.*,[25]studied the Eupatorin present in *Artemisia annua* which revealed a moderate cytotoxic effect on MK-1 (human gastric adenocarcinoma), HeLa (human uterus carcinoma), B16F10 (murine melanoma), and 26-L5 (murine colon cancer) cell lines. Interestingly, the same compound was totally inactive against P-388 (lymphatic leukemia) cell line. Eupatorin inhibited the growth of MDA-MB-468 (human breast carcinoma) cell line in a

dose dependent manner with an $IC_{50}$ of 0.5 μM, eupatorin showed only weak inhibitory effect against MCF- 10 (normal mammary tissue) cells. Eupatorin was found to be metabolized to the flavone cirsiliol and two other unidentified metabolites by a CYP450 enzyme. The enzyme is present in MDA-MB 468 cells, but not expressed in MCF-10A healthy mammary cells. A similar activation of eupatorin by a CYP450 enzyme has also been observed in MCF-7 (human breast adenocarcinoma) cells104.

Tungmunnithum *et al.,*[26]investigated quercetin another common flavones of Asteraceae it has been extensively studied for its biological activities including anticancer activity. Quercetin inhibited the growth of MCF-7 breast cancer cell line with an $IC_{50}$ value of 5.2 mg/mL. Quercetin has been shown to inhibit protein kinase C and to bind to type-II estrogen binding sites. Interestingly, structurally similar flavonoids, myricetin and epicatechin, did not show significant inhibitory activity against MCF-7, indicating some specific structure-related interactions between quercetin and its potential cellular target(s).

# CHAPTER – 1

# INTRODUCTION

Cancer is the growth of tissues characterized by the uncontrolled division of cells and they severely affect the human population. According to the World Health Organization, cancer is the second leading cause of death globally and was responsible for an estimated 9.6 million deaths in 2018 [27]. Globally, about 1 in 6 deaths is due to cancer. In India, it is estimated that around 2.25 million people are suffering from different types of cancer according to the National Institute of Cancer Prevention and Research [28].

Cancer's imperative features are alteration in the mechanisms that control the cell proliferation and differentiation. Unlike normal cell behavior, cells with neoplastic transformation endure repeated cycles of proliferation and metastasize to distant organs due to expression of cell surface antigens [29]. This type of tumor cells is known as malignant tumor which can invade normal tissues surrounding the body through blood or lymphatic vessels. Malignant tumor is hardly encapsulated and has abnormal morphology where their tissue origin may not be recognizable. However, the migration of cancerous cells to other site to develop secondary cancer is dependent on the interaction of metastatic cells with different organ environments like rate of blood flow (angiogenesis), vascular anatomy and number of cells delivered to secondary organ [30]. Since neoplastic cells are biologically heterogenous which contain diverse population of tumor cells, this feature orchestrates the invasion, via extracellular matrix, intravasation, endurance in a blood or lymphatic vessel, extravasation into a distant site, to cause illness and eventually, death of the patient[31].

Chemotherapeutic drugs are a novel treatment modality with the aim of reversing or halting carcinogenesis. In case of disseminated cancers (lymphoma, for example), chemotherapy is the

sole therapeutical approach to surgical procedure and radiotherapy. Ideal anticancer agents would lead to absolute remissions of cancer cells without affecting normal cells. Regrettably, there is not any agent available to meet this standard, as current chemotherapy agents are not able to distinguish between eradiating the cancer and normal cells [32]. Rather, these drugs weigh benefits against toxicity in a search for a favorable therapeutic index. The common adverse effect from chemotherapy procedure includes gastrointestinal problems (nausea, vomiting, and diarrhea), bone marrow suppression, infertility, anemia, and alopecia [33].

Despite dramatic improvements in patient survival and drug tolerability, several drugs may present life threatening toxicities such as organ damage and hematological toxicity. Now, chemotherapy is more common as palliative therapy rather than therapeutical therapy that functions to provide temporary relief of the symptoms and signs of cancer and complete enhancement of life quality [34]. Cancer growth eradication in absence of adverse effects is acknowledged as a vital goal for the development of cancer therapy [35].Owed to the current preoccupation with synthetic chemistry's safety and efficacy, the dependence on the natural products is becoming colossal. Natural products being synonymous with "secondary metabolite" are identified from herbal plants, with substantial part in the prevention and therapy of cancer management. A vast range of structurally novel chemo types with potent and selective biological activity can serve as chemical models or templates to design, synthesis, and semi synthesis of novel substances for the treatment of various human disorders [36].

Out of 155 anticancer drugs approved in western medicine, 47% were classified as natural products. Plants, animals, marine organisms and microorganisms hold tremendous

amount of secondary metabolites as potential anti-cancer agents. Thus, a significant number of plant-sourced agents and the mechanism of interaction between many phytochemicals and cancer cells have been extensively focused for the treatment of cancer [37].

Traditional Indian medicinal plants have been in use from time immemorial and their utility has been increasing day by day in the present scenario. Naturally obtained compounds are considered safer and easily biodegradable than synthetic compounds and the problem of drug resistance observed in synthetic drugs is also reduced [38]. Plants and plant products represent a source of leads for many pharmaceutical products, phytochemical compounds and secondary metabolites in plants have been used in treating a number of human ailments. Drugs obtained from medicinal plants comprise 25% of total drugs in developed countries and about 80% in developing countries [39]. Cancer is the leading cause of mortality across the world and the failure of conventional chemotherapy to effect major reduction in the mortality indicates that new approaches are essentially needed [40]. An extremely promising strategy for cancer prevention by only method is chemotherapy, which is defined as the use of synthetic or natural agents to block the development of cancer in humans [41]. A variety of bioactive compounds and their derivatives have been shown to inhibit carcinogenesis in a number of experimental system involving initiation, promotion and progression [42]. Plants, vegetables and herbs are used as folk drug and traditional medicines have been accepted currently as one of the main sources of cancer chemoprevention drug [43].

Development of cancer is the result of a continuous process that occurs several years during that time there is a damage of numerous regulatory genes eventually resulting in premalignant and then metastatic stages [44]. Cancer is a heterogeneous disease composed of

complex genetic changes, driving uncontrolled growth and metastatic spread. Reactive oxygen species and reactive metabolic intermediates generated from various chemical carcinogens are known to play an important role in cell damage and in the initiation and progression of carcinogenesis [45].

The intake of natural compound has been associated with reduced risk of cardiovascular disease, diabetes and other diseases related to aging and cancer. Cancer is the second largest cause of death in the world and there is no completely effective medicine to treat most cancers, further more today many cancer treatments are very expensive. During the 1960's national cancer institute (USA) began to screen plant extracts with antitumor activity [46]. As rich sources of novel anticancer drugs, natural compounds isolated from medicinal plant have received increasing interest ever since [47].

Leukemia is one of the most common malignancies causing death worldwide. Although chemotherapy is the standard method of treatment for patients, it has not been fully effective. Therefore, development of new agents is still important to reduce the rate of mortality [48]. The HL-60 cell lines, derived from a single patient with acute promyclocytic leukemia (APL), provided a unique *in vitro* model system for studying the cellular and molecular events involved in the proliferation and differentiation of normal and leukemic cells of the granulocytes /macrophage lineage [49].

The important characteristics of this unique cell lines, with particular emphasis on HL-60 proliferation, differentiation and cellular oncogenes expression [50]. HL-60 cells also exhibit genetic abnormalities in cellular oncogenes and these abnormalities, most likely also play a role

in the unusual ability of myeloid leukemia cells to proliferate continuously *in vitro*. MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was performed to study cytotoxic activity, whereas Comet assay was undertaken to study genotoxic activity and apoptotic activity was done for the necrosis study.

Cancer is a multi-step disease incorporating physical, environmental, metabolic, chemical and genetic factors [51]. Breast cancer is the most commonly occurring cancer in women, comprising almost one-third of all malignancies [52]. It accounts for approximately 25% of all female malignancies with a higher prevalence in developed countries. Breast cancer is the second leading cause of cancer-related death among females in the world [53].

Development of breast cancer cell targeting drug without affecting the normal cells is a challenging task in the field of cancer drug discovery. The limited success of clinical therapies including radiation, chemotherapy, immunomodulation and surgery in treating cancer, as evident by the high morbidity and mortality rates, which also can produce severe side effects such as bone marrow depression, lecukopenia, anaemia, alopecia, and hyperuricaemia, teratogenicity, carcinogenicity, and also its reduced spermatogensis in men, amenoria in women because of this indication there is an imperative need of new cancer management. Herbal Plants have long been shown to be excellent and reliable sources for the development of novel anti-cancer drugs [54]. Use of natural products and its derivatives in the development of anticancer drugs are increasing all over the world because of lesser side effects as compared to synthetic drugs[55,56,57] as more than 60% of the chemotherapeutic drugs are developed from plants and their derivatives. Medicinal plants are potential sources of natural products exhibiting anti-proliferation and anti-metastatic properties [58].

Cancer is a major public health problem in the world. Breast cancer is the second leading cause of cancer deaths among women [59]. Chemotherapy is one of the commonly-used strategies

in breast cancer treatment. This therapy is usually associated with adverse side effects, ranging from nausea to bone marrow failure. Therefore, finding natural compounds from plants may provide an alternative cancer treatment.

Breast cancer is the second largest cancer after lung cancer in the world and the most common malignancy among women [60]. Breast cancers can be classified by stage, pathology, grade, and expression of estrogen receptor (ER), progesterone receptor (PR), or human epidermal growth factor receptor (Her2/neu). MDA-MB-468 cells are characterized as ER-, PR-, and Her2/neu-negative/basal-A mammary carcinoma [61]. MDA-MB-468 cells were derived from the pleural effusions of 51-year-old female patients. MDA-MB-231 cells were derived from a Caucasian female, while MDA-MB-468 cells were derived from an African American female [62,63]. The search for cancer drugs from natural sources started with discovery of Podophyllotoxin in late 1960s, which further lead to the discoveries of vincristine, vinblastine, campthothecin and taxol [64]. Nature counts more than 1000 species of plant varieties which possess significant anti-cancer properties. Taxol, one of the most outstanding agents has been found beneficial in the treatment of refractory ovarian, breast and other cancers. Podophyllotoxin, synthetic modification of this molecule led to the development of Etoposide known to be effective for small cell cancers of the lungs and testes [65].

The discovery of medicinally important herbs and their mechanism of action would provide an alternative and effective treatment towards the cancer prevention. Since decades, plethoras of drugs have been developed from potent compounds which are isolated from medicinal plants. Traditional medicine and knowledge of Ayurveda help in the discovery of new

drug having high activity and low toxicity for cancer therapy. Initial research focuses on the isolation of bioactive lead compounds, chemical modification and improving other pharmacological profiles.

Cancer is characterized by a rapid and uncontrolled formation of abnormal cells, which may mass together to form a growth or tumour, or proliferate throughout the body, initiating abnormal growth at other sites. If the process is not arrested, it may progress until it causes the death of the organism [66]. Oral cancer can result from poor life style choices such as smoking tobacco and consumption of alcohol that are considered as major risk factors in the development of this type of cancer [67,68]. Tobacco contains known carcinogens such as N'-nitrosonornicotine and aromatic hydrocarbon benzo-pyrene, N-nitrosomine (4-nitrosomethylamino)-1-(3-pyridyl)-1-butanone (NNK). These compounds have been linked to oncogenesis capable of inducing tumors of the oral and nasal cavities, lungs, esophagus and pancreas [69]. Biological agents can also cause oral cancer. Genetic predisposition is another important risk factor in the development of oral cancer. Evidence shows that certain individuals inherit genetic dispositions that result in the inability to metabolize carcinogens as well as repair DNA damage thus causing cancer. The *human papilloma virus* was able to cause oral carcinoma [70]. Treatment of oral cancer has been achieved through the use of either surgery or radiation or both depending on the severity of the disease. These treatment options result in side effects such as vomiting, nausea, hair loss, fatigue, mouth sores and complications like mucositis [71].

Apoptosis or programmed cell death is a highly organized physiological process to eliminate damaged or abnormal cells. It also plays a major role in embryogenesis where apparently normal cells undergo apoptosis. It is involved in maintaining homeostasis in multicellular organisms [72]. Traditional medicine refers to the application, approach, knowledge and belief in incorporating plant and animal based properties in remedies for the purpose of treating or preventing disease as well as to maintain the well-being of an individual. Herbal remedies have been used to cure a variety of disorders like diabetes, sexual malfunction, and urinary tract infections in females, cardiovascular diseases, and weight control and used to cure many other ailments. Plants have a long history of use in the treatment of cancer. Plants have played an important role as a source of effective anti-cancer agents and it is reported that over 60% of currently used anti-cancer agents are derived from natural sources, including plants, marine organisms and micro-organisms [73].

# CHAPTER – 2

# STRUCTURAL ELUCIDATION OF PHENOLIC COMPOUND

## About Plant Species

The genus *Pandanus* of the Pandanaceae family comprises about 700 species distributed world wide. Various Pandanus species are also used in folk medicine [74].

Pandan is said to be a restorative, deodorant, indolent and phylactic, promoting a feeling of wellbeing and acting as a counter to tropical lassitude. It may be chewed as a breath sweetener or used as a preservative on foods. It is also said to have healthful properties, including antiviral, anti-allergy, antiplatelet, anti-inflammatory, antioxidant and antitumor [75]. The medicinal action of the essential oil yielded by the screw pine's highly scented flowers to be useful in headaches, earaches, and as a liniment for rheumatic pains. The distilled water made from, the flowers are used for inducing perspiration. It is also prescribed as a stimulant and an antispasmodic. The flowers themselves are powdered and included in medicines, which are either sniffed like snuff or smoked for asthma and other bronchial infections [76].

### Taxonomical Identification

Traditional Indian medicinal plant was identified and authenticated as *Pandanus odorattissimuss* (specimen No. RRCBI- 12749) by Dr. N. Siddmallayya, Research Officer. National Ayurveda Dietetics Research Institute (NADRI), Government of India, Jayanagar, Bangalore, Karnataka, India.

**Structural Elucidation of Compound**

The test compound isolated from the leaves of *Pandanus odorattissimus* L. was subjected to IR, NMR and Mass Spectroscopy. The spectral data was obtained is of immense use in the detection of the functional groups and further in elucidation of structures [77].

**Infra Red Spectrum (IR)**

The infrared spectra of phenolic compound was obtained by KBR discs on Perkin elmer RX1 spectrometer in the wave number (cm-1) in the range of 2300-200 cm-1 was recorded as the inverted peaks.

The infra IR spectrum of compound showed peaks at 3434, 2924, 2859 cm- 1 due to -$OCH_3$-$OCH_3$ and -$OCH_3$groups near to ester group respectively. The peak exhibited at 1747 is presence of cm-1 due to -C=0 group of esters.

**1H Nuclear Magnetic Resonance Spectrum (1H NMR)**

The 1H NMR spectra of phenolic compound was recorded in JEOL Model GS x 400 spectrophotometer $CdCl_3$ (denatured methanol) was the solvent used. NMR was recorded in the Bruker AMX 400 NMR Spectrometer using TMS (Tetramethylsilane) as an internal standard at 500. 124 MHz (1H) and 270C or 300 K. The chemical shifts were recorded in d (ppm) based either on d TMS = 0 and the coupling constants or in hertz.

1H NMR spectrum showed five singlets at 7.07, 7.23, 7.75, 8.09, 8.51 d (ppm) resonated to multiple of five protons of aromatic systems. The d 5.07 two protons of furan rings. The d 3.40 has single furan compound. The d 2.51 resonated to singlet of one proton resonated to ester group. The compound singlet peak at d 8.51 (1*H, br s*) was identified as phenolic hydroxy. The identification was further confirmed by 1H NMR spectral data available in literature. **(Spectra-2)**.

**Mass Spectrum (MS)**

The fast atom bombardement – Mass spectra of the purified compound was recorded on a Schimadzu instrument at 70 *eV* by direct in let method. At room temperature ($27^0$C), M-nitrobenzyl alcohol (NBA) was used as the matrix unless specified otherwise. The matrix peaks appeared at m/z 136, 137, 154, 289 and 413 in the absence of any metal ions. If metals ions such as Na+ are present, these peaks may be shifted accordingly. The positive FAB- MS spectra were obtained m/z verses relative abundance after taking 2- 4 scans.

The mass spectrum displayed a molecular ion [M+] peak at m/z 414 (15%) corresponding to molecular formula ($C_{23}H_{26}O_7$) together with fragmentation at m/z 324 (8%), m/z 154 (55%) and m/z 127 (35%) **(Spectra-3)**.

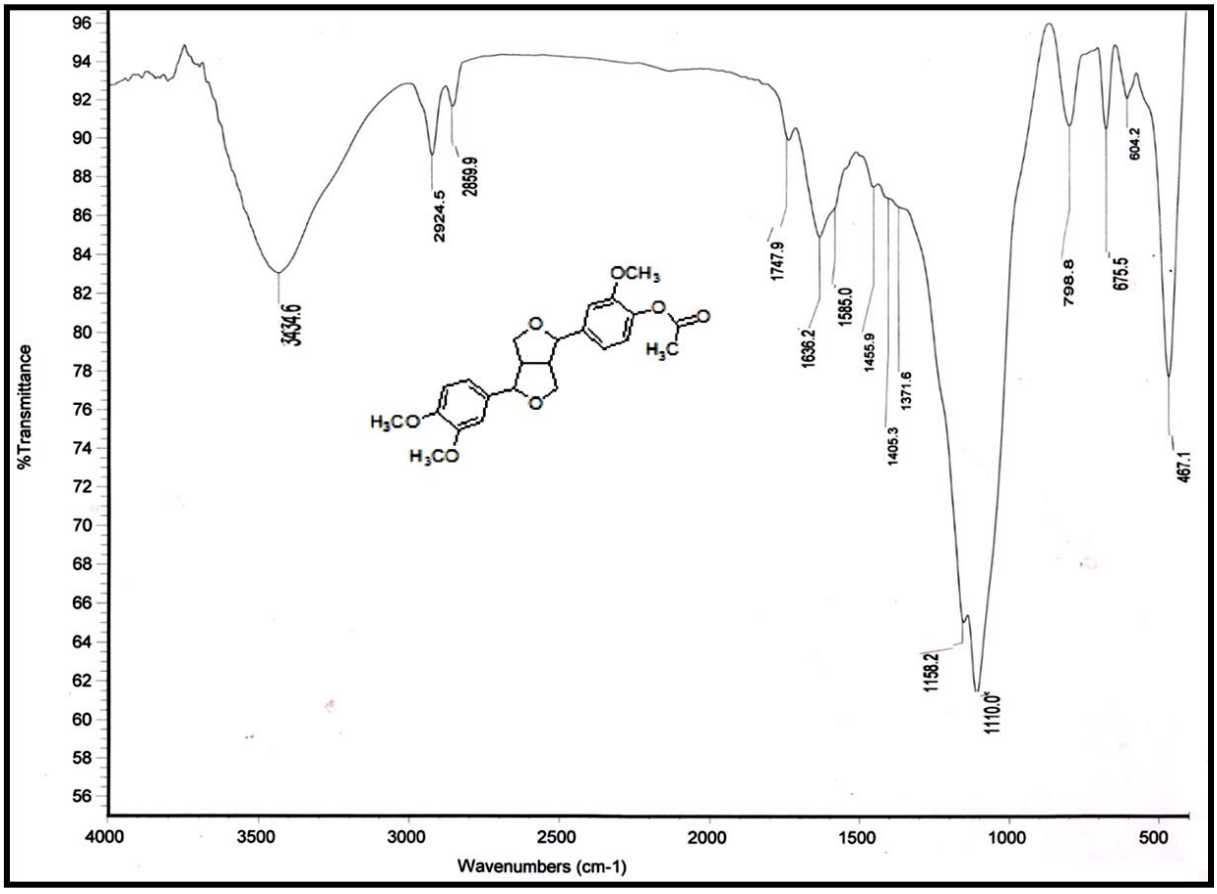



**Image - 2.1 : Infra Red Spectrum of phenolic compound**

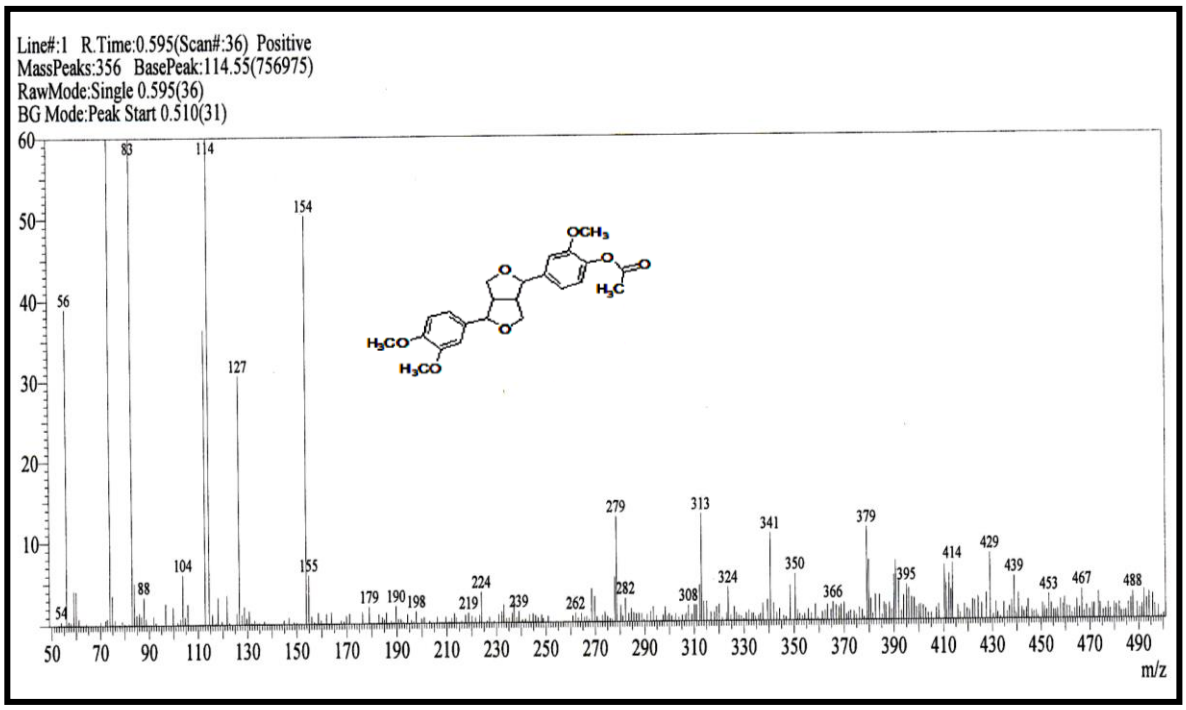



**Image -2.2: 1H NMR of phenolic compound**

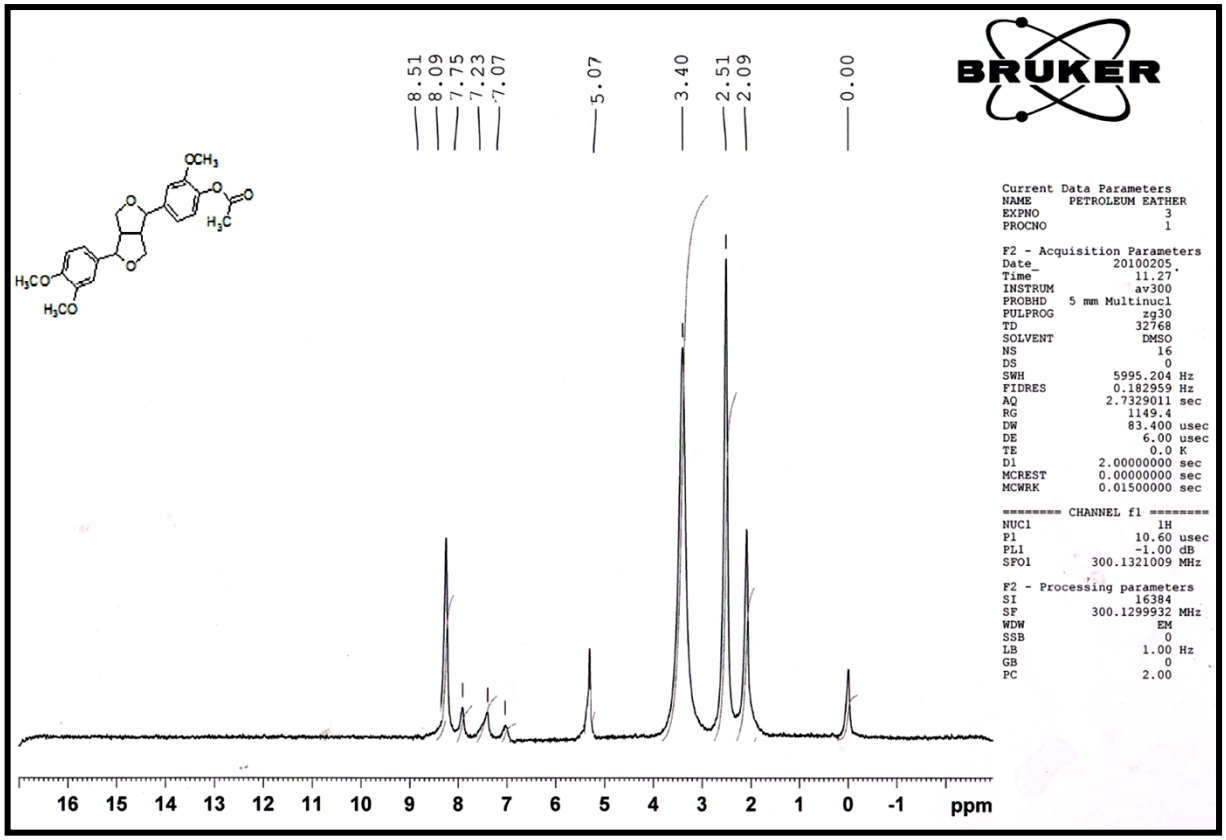



**Image -2.3: MS of phenolic compound**

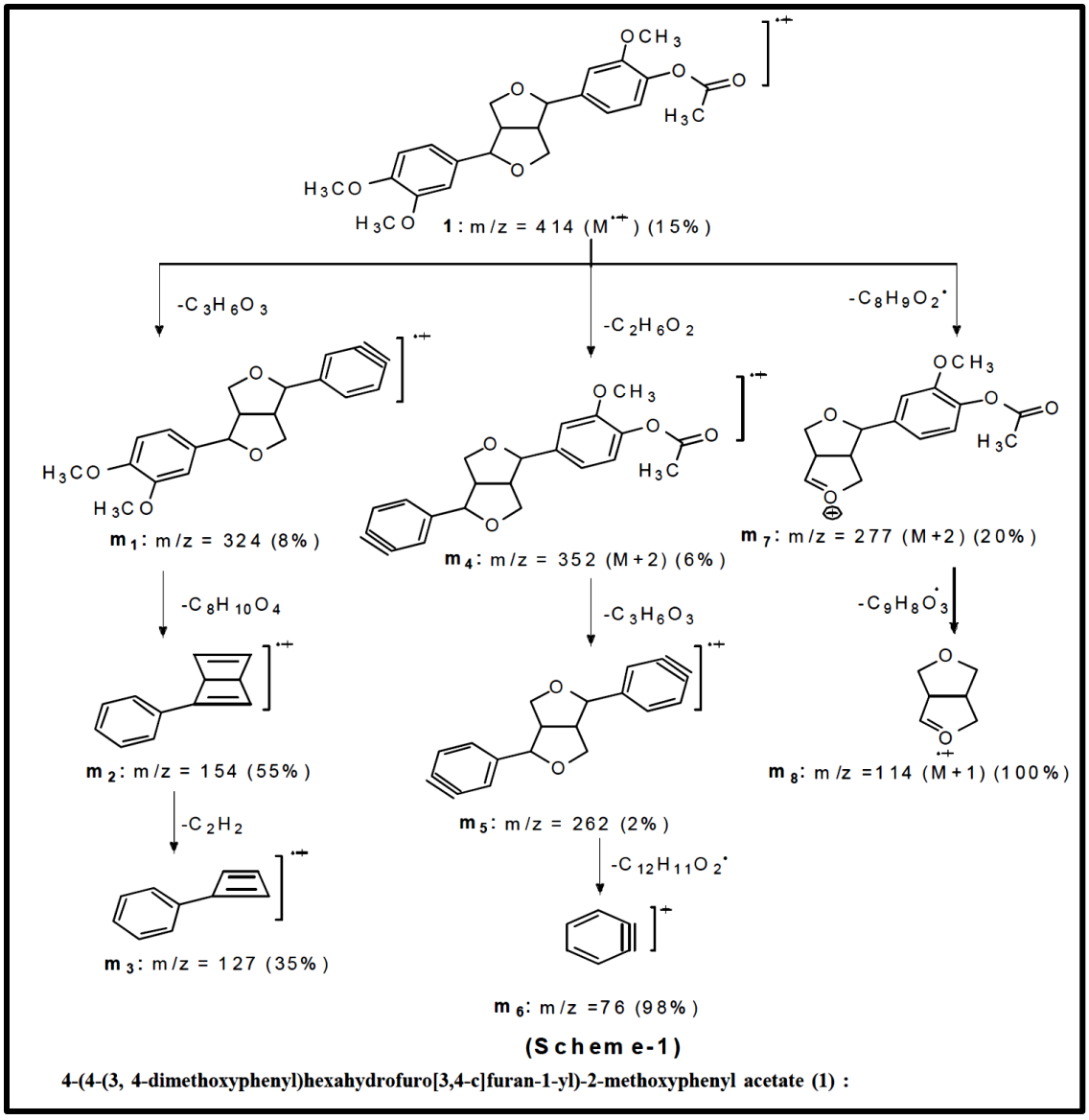



**Image - 2.4:** Scheme of structural elucidation of phenolic 4-(4-(3,4-dimethoxyphenyl) hexahydrofuro[3,4-c]furan-1-yl)-2-methoxyphenyl acetate compound.

**Structure of Phenolic Compound**

The IR spectrum of *Pandanus odorattissimus* L. exhibited absorption band at 3434, 2924 and 2859 cm-1due to -$OCH_3$, -$OCH_3$ and -$OCH_3$ groups respectively near to ester group. The band exhibited peak at 1747 due to C=O group of ester. The 1H NMR spectrum δ 7.07 - 8.51 resonated to multiplet of five protons of aromatic systems. The δ 5.07 resonated due to two protons of furan rings and δ 3.84 due to single furan compound. The δ 2.51 resonated to singlet of one proton resonated to ester group. The mass spectrum of compound displayed molecular weight at M/Z 414 (15%) corresponding to molecular weight. Based on above studies the structure of compound was proposed as 4-(4-(3,4-dimethoxyphenyl)hexahydrofuro[3,4-c]furan-1-yl)-2-methoxyphenyl acetate, which is mentioned as phenolic compound hence forth throughout the text.

# CHAPTER – 3

# *IN VIVO* CYTOTOXIC STUDIES OF PHENOLIC COMPOUND

## 3.1. Induction of Tumour, Determination of Tumour, Size, Hematological Parameters in Mice

### Materials and Methods

Swiss albino mice of 8-9 weeks old, weighing 25 ± 2 gm from inbred colony were used for the study and were maintained in animal house at MAM college of pharmacy, Gulbarga University, Gulbarga, India with a 12 h light/12 h dark cycle, fed with standard pellet food as described by Central Food and Technological Research Institute (CFTRI, Mysore) formula and provided tap water ad *libitum*.

### Determination of Median Lethal Doses ($LD_{50}$)

The $LD_{50}$ values were estimated by conducting the acute toxicity study in Swiss albino mice [79]. The test compound was dissolved in 3% dimethyl sulfoxide and administered intraperitoneally to different groups with increasing concentration. Eight animals were placed in each group. Mortality was determined after 24 hours of treatment. The dose at which 50% of the mice survived was considered as the $LD_{50}$ value of the compound.

### Tumor Cells

Transportable tumor induced by (EAC) cells were used in this experiment. The initial inoculums of EAC cells were procured from National Centre for Cell Science (NCCS), Pune, India. The EAC cells were then propagated in our laboratory biweekly through intra peritoneal (i.p.) injections of $3 \times 10^6$ cells (freshly drawn from a donor Swiss albino mouse, bearing 6 to 7 day old ascites tumor cells).

**Cell Growth Inhibition**

Five groups of mice (8 in each group) weighing 25 g to 30 g were selected for the experiment. A total of $136 \times 10^4$ EAC cells were inoculated into each group of mice on day zero. The treatments were initiated after 24 h of tumor cell inoculation and continued for 7 days. Groups 1 to 3 received the test phenolic compound 4-(4-(3,4-dimethoxyphenyl) hexahydrofuro[3,4-c]furan-1-yl)-2-methoxyphenyl acetate at doses of 10, 25, and 50 mg/kg (i.p.), respectively. Group 4 received Vinblastine at a dose of 0.5 mg/kg (i.p.), and Group 5 was used as the control. On 8th day all the mice of each group were sacrificed and total intraperitoneal tumor cells were harvested by using normal saline (0.98%). Viable cells were identified by trypan blue and counted with hemocytometer. Total number of viable cells in every animal of treated groups were compared with controlled (untreated EAC inducing mice) group.

**Average Tumor Weight and Mean Survival Time**

Five groups of mice (8 in each group) were used for the experiment. A total of $136 \times 10^4$ EAC cells were inoculated in each mouse on day zero. The treatment was initiated after 24 h of tumor cell inoculation and continued for 10 days. Gravimetric changes in each mice were recorded daily and increase in tumor weight was monitored. The host survival was recorded and expressed as mean of survival time in days. Percent increase in the lifespan was calculated by using following formula: Mean survival time (MST) = Day of 1st death + Day of last death / 2. Percent increase of lifespan (% ILS) = (MST of treated group) – (MST of Control group) X 100 / MST of Control group

**Hematological Parameters in Normal and Tumor Bearing Mice**

The effects of the test compound on hematological parameters were studied in both normal and tumor bearing mice [80]. For the tumor bearing mice, treatment was initiated after 24 h of tumor transplantation and continued for 10 days. Hematological parameters in normal mice were studied using a similar method used for the tumor bearing mice. Blood samples were collected from tail vein of mice from each group on days 7, 14, 21 and 28 for the studies.

**Effects of the Phenolic Compound on Normal Peritoneal Cells**

Effects of phenolic compound on the normal peritoneal cells were determined by counting the total peritoneal cells and the number of macrophages [81]. Four groups of mice (8 in each group) were used in the experiment. Groups 1 to 3 were treated with phenolic compound 4-(4-(3,4-dimethoxyphenyl)hexahydrofuro[3,4-c]furan-1-yl)-2-methoxyphenyl acetate at dose level of 10, 25, and 50 mg/kg (i.p.), respectively, for three consecutive days. Group 4 was used as control. After 24 h of last treatment, each animal was injected with 5 mL normal saline (0.98%) into the peritoneal cavity and was sacrificed. Intraperitoneally exuded cells and the number of macrophages were counted with 1% neutral red by using a hemocytometer.

**Statistical Analysis**

Data were expressed as the Mean ± S. E. M. The data were analyzed statistically using SPSS Version 10.

# RESULTS

## $LD_{50}$ of compound

The lethal dose of phenolic compound (4-(4-(3, 4-dimethoxyphenyl)hexahydrofuro[3, 4-c] furan-1-yl)-2-methoxyphenyl acetate) was found to be 100 mg/kg for intraperitoneal treatment of Swiss albino mice.

## Body Weight

Treatment of mice with phenolic compound 4-(4-(3, 4-dimethoxyphenyl) hexahydrofuro [3, 4-c] furan-1-yl)-2-methoxyphenyl acetate resulted in body weight reduction in experimental mice both in the treated and control group at the beginning of the experiment (day 0) and on the final day prior to sacrifice. The body weight was significantly ($P< 0.01$) reduced when the EAC implanted animals were treated with phenolic compound at the dose level of 10, 25 and 50 mg/kg and standard drug 0.5mg/kg (Vinblastine) for 7 days (Table- 3.1.1).

## Tumor Weight

The mice were dissected and the ascitic fluid was collected from the peritoneal cavity. The tumor weight is calculated from the difference in weight of mice before dissection and after collection of ascitic fluid after dissection. The tumor weight was significantly reduced 10mg/kg ($P<0.05$), phenolic compound at concentration of 25 and 50 mg/kg ($P<0.001$) reduced when experimental group were compared with EAC bearing group and standard drug at 0.5mg/kg ($P<0.001$) (Vinblastine) for 7 days (Table- 3.1.1).

**Inhibition of Cell Growth**

The treatment of mice with phenolic compound 4-(4-(3, 4-dimethoxyphenyl) hexahydrofuro [3, 4-c] furan-1-yl)-2-methoxyphenyl acetate resulted in a cell growth inhibition by 75.02% ($P$>0.001), 63.36% ($P$>0.01), and 27.28 ($P$>0.01) % at the doses of 50, 25, and 10 mg/kg (i.p.), respectively. Standard drug Vinblastine at 0.5 mg/kg (i.p.) exhibited inhibition of 86.12% ($P$>0.001) when compared to that of control (Table- 3.1.2).

**Survival Time, Day and Life Span**

All the treated mice showed significant effect on the survival time of the EAC bearing mice and acted as anticancer drug. (Table- 3.1.2) represents the effect of test compound on survival time of EAC bearing mice at different doses.

The experimental mice showed significant increase in lifespan by 29.12%, 58.78%, and 80.51% for the doses of 10, 25, and 50 mg/kg (i.p.), respectively. Evidently, the survival time of mice increased with increasing dose of test compound. Whereas, Vinblastine at 0.5 mg/ kg (i.p.) showed increase in the life span by 86.51% compared with the control. Increasing percentage of a lifespan of mice at 50 mg/kg (i.p.) of phenolic compound is quite comparable to the effects of the standard anticancer agent Vinblastine (0.5 mg/kg). The treatments of animals previously inoculated with EAC cells with test compound resulted in tumor growth inhibition.

**Enhancement of Peritonial Cells and Macrophages**

Effect of the test compound on normal peritoneal cells is shown in (Table- 3.1.4). Treatment of mice with the phenolic compound at increasing doses resulted in an increase of normal peritoneal cells and macrophages significantly ($P<0.05$) when compared to standard drug vinblastine.

**Hematological Study**

Effects of the phenolic compound on hematological parameters in tumor bearing mice are shown in (Figure – 3.1.1 to 3.1.6). Hematological parameters varied from their normal values along with tumor growth. Hemoglobin content and RBC counts were found to be decreased, whereas, the WBC counts increased after inoculation of EAC cells. After treatment with test compound at the previously specified doses, parameters were restored moderately at a high concentration of phenolic compound.

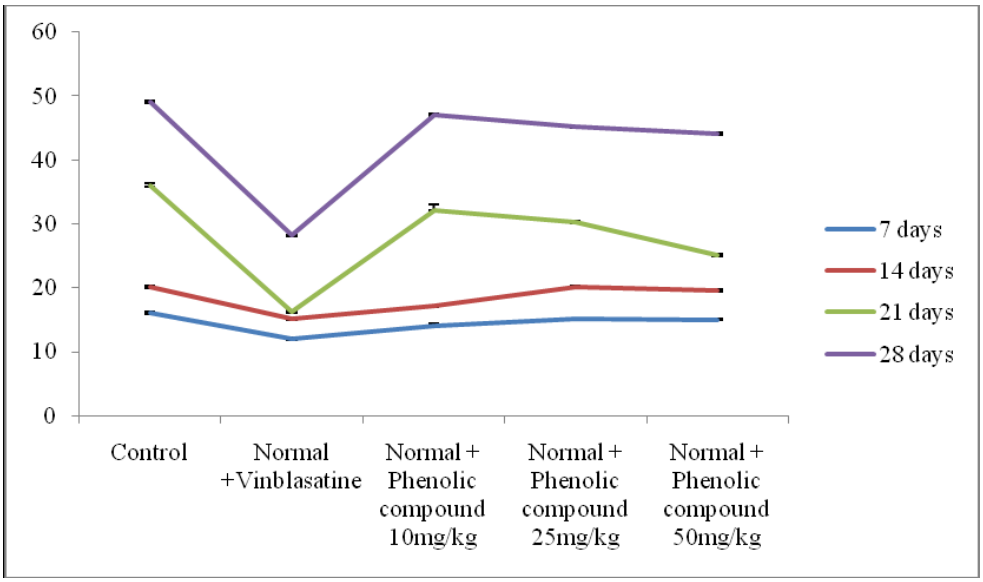



**Fig - 3.1.1.** Effect of phenolic compound RBC of normal mice on days 7, 14, 21, and 28. Data are expressed as mean of the results from 8 mice.

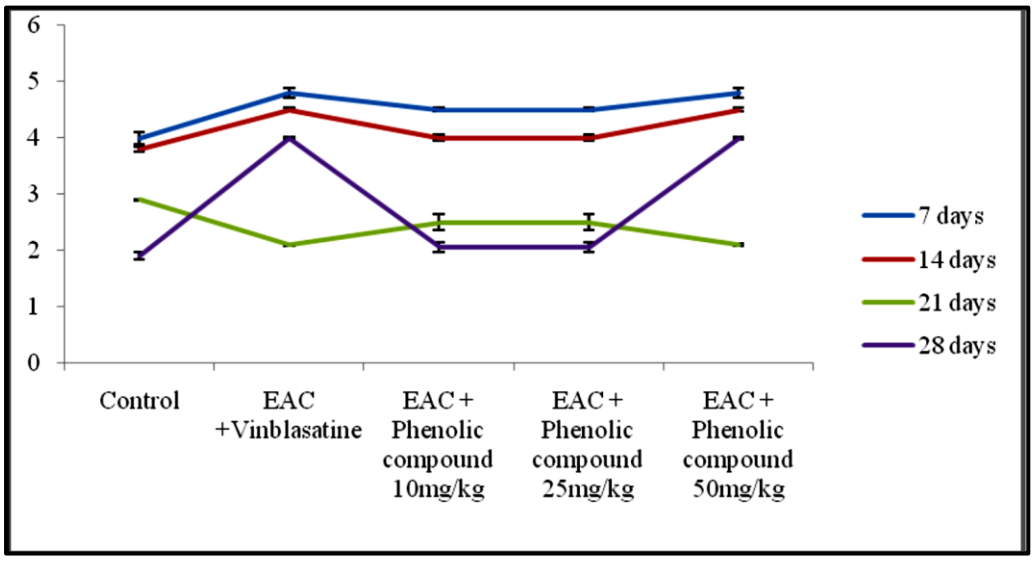



**Fig - 3.1.2.** Effect of test compound on RBC of EAC bearing mice on days 7, 14, 21, and 28. Data are expressed as mean of the results of 8 mice.

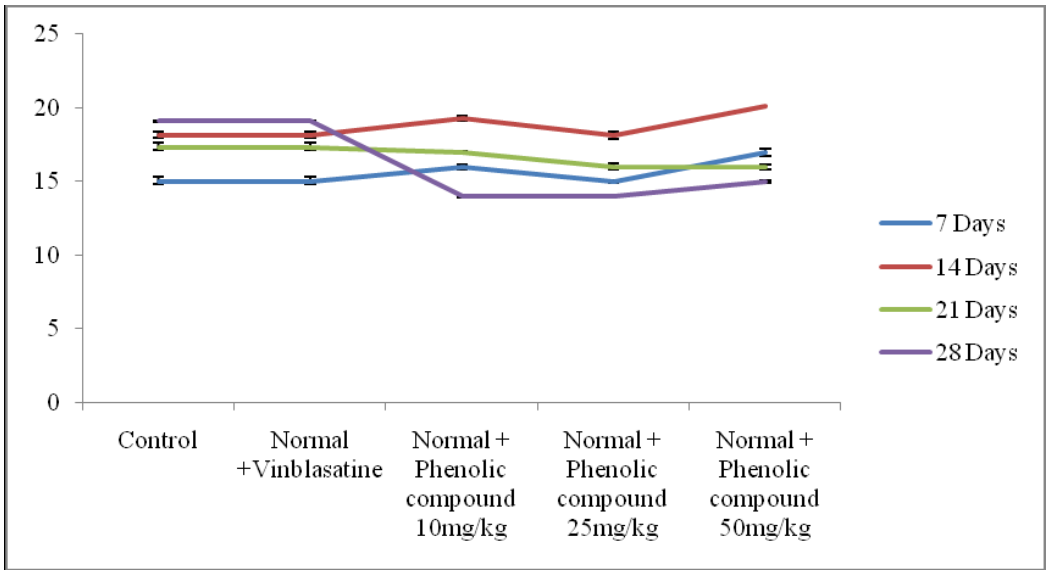



**Fig - 3.1.3.** Effect of phenolic compound on WBC of normal mice on days 7, 14, 21, and 28. Data are expressed as mean of the results from 8 mice.

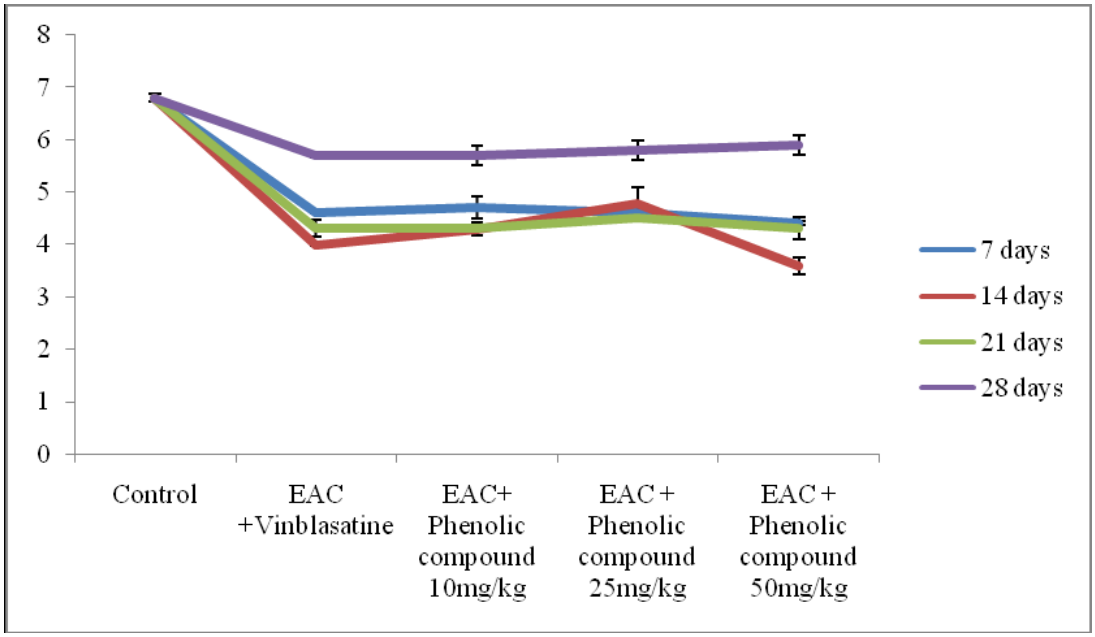



**Fig - 3.1.4.** Effect of phenolic compound on WBC of EAC bearing mice on days 7,14, 21, and 28. Data are expressed as mean of the results from 8 mice.

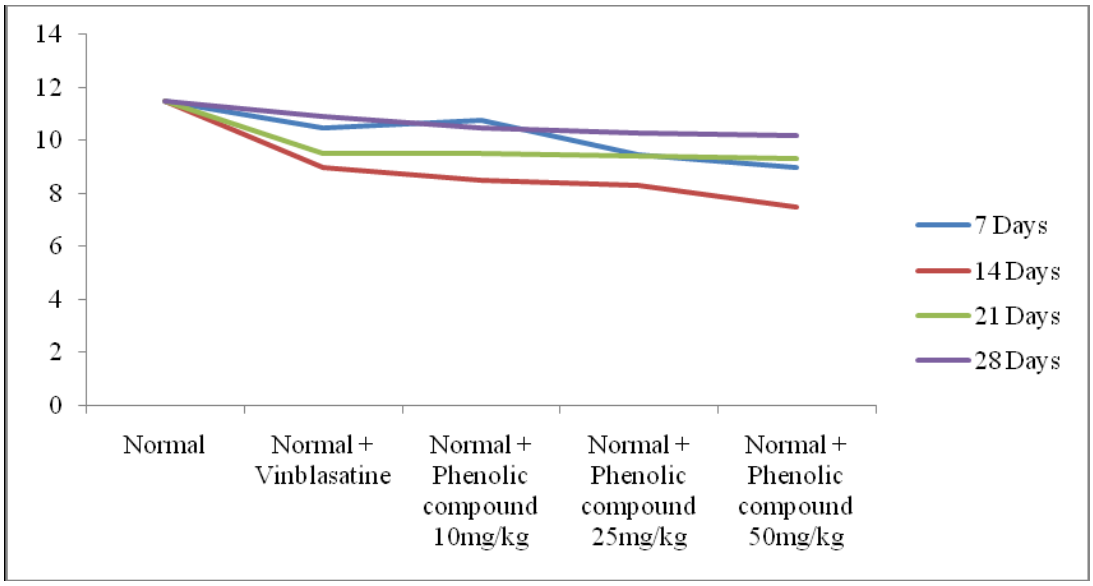



**Fig - 3.1.5.** Effect of phenolic compound on hemoglobin content of normal mice on days 7, 14, 21, and 28. Data are expressed as mean of the results from 8 mice.

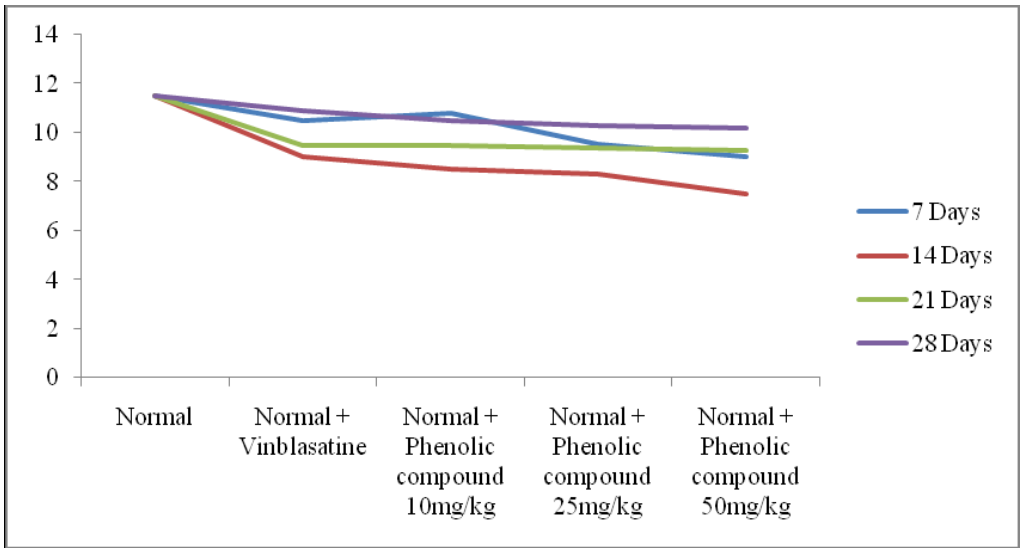



**Fig - 3.1.6**. Effect of phenolic compound on the hemoglobin content of EAC bearing mice on days 7, 14, 21, and 28. Data are expressed as the mean of the results from 8 mice.

**Table - 3.1.1.** Effect of test (phenolic) compound (4-(4-(3,4-dimethoxyphenyl)hexahydrofuro[3,4-c]furan-1-yl)-2-methoxyphenyl acetate) on body weight and average tumor weight in mice

| **Treatment Group** | **Dose (mg/kg (i.p.))** | **Body Weight (gm)** | **Average Tumor Weight (gm)** |
|---|---|---|---|
| **Normal** | **---** | **23. 25** ± 0.06 | **--** |
| Control (EAC cell bearing mice) | -- | 19.22 ± 0.12 | 3.15 ± 0.04 |
| Vinblastine | 0.5 | 18.59 ± 0.09* | 1.06 ± 0.01*** |
| Phenolic compound | 10 | 17.0 ± 0.02** | 2.10 ± 0.02* |
| Phenolic compound | 25 | 18. 06 ± 0.05* | 1.53 ± 0.09*** |
| Phenolic compound | 50 | 18.36 ± 0.13* | 1.13 ± 0.03*** |

Number of mice in each case was eight ($n$=8). Results are shown as mean ± SE. Significant values are *$P$ < 0.05, **$P$ < 0.01, and ***$P$ < 0.001.

**Table - 3.1.2.**Effect of phenolic compound on EAC cell growth inhibition in mice.

| **Treatment Group** | **Dose (mg/kg (i.p.))** | **No. of EAC cells in mouse on day 8 after inhibition of cell** | **Tumor cell inoculation growth %** |
|---|---|---|---|
| Control (EAC cell bearing mice) | -- | $2.517\pm0.182)\times10^{7}$ | |
| Vinblastine | 0.5 | $(0.297\pm0.012)\times10^{7}$*** | 86.12 |
| Phenolic compound | 10 | $(1.766\pm0.004)\times10^{7}$** | 27.28 |
| Phenolic compound | 25 | $(0.962\pm0.017)\times10^{7}$** | 63.36 |
| Phenolic compound | 50 | $(0.498\pm0.007)\times10^{7}$*** | 75.02 |

Number of mice in each case was eight ($n$=8). Results are shown as mean ± SE. Significant values are *$P$ < 0.05, **$P$ < 0.01, and ***$P$ < 0.001. Σ Survival time (days) of each mouse in a group Total number of mice MST of treated group MST of control group (-1 ) × 100.

**Table – 3.1.3.** Effect of the phenolic compound on the survival time of EAC bearing mice.

| **Treatment Group** | **Dose (mg/kg (i.p.))** | **Mean survival time, day (Mean±SE)** | **Increase of life span (%)** |
|---|---|---|---|
| Control (EAC cell bearing mice) | -- | 20.18±0.96 | |
| Vinblastine | 0.5 | 41.15±0.98** | 86.51 |
| Phenolic compound | 10 | 30.25±2.18* | 29.12 |
| Phenolic compound | 25 | 34.31±1.04** | 58.78 |
| Phenolic compound | 50 | 43.13±2.24* | 80.71 |

Number of mice in each experiment was Eight ($n$=8). Results are shown as mean ± SE. Significant values are *$P$ < 0.05 and **$P$ < 0.01.

**Table - 3.1. 4.** Effect of phenolic compound on the enhancement of normal peritoneal cells in mice.

| **Treatment Group** | **Dose (mg/kg (i.p.))** | **Macrophages (cells/mL)** | **Total peritoneal cells (cells/mL)** |
|---|---|---|---|
| Control (EAC cell bearing mice) | -- | | |
| Vinblastine | 0.5 | $(1.78\pm0.42)\times10^6$ | $(8.26\pm0.58)\times10^6$ |
| Phenolic compound | 10 | $(2.12\pm0.46)\times10^6$* | $(9.18\pm0.36)\times10^6$* |
| Phenolic compound | 25 | $(2.43\pm0.28)\times10^6$* | $(9.67\pm0.24)\times10^6$** |
| Phenolic compound | 50 | $(2.61\pm0.34)\times10^6$* | $(10.14\pm0.32)\times10^6$* |

Number of mice in each group was four ($n$=4). Results are expressed as mean ± SE. Significant values are *$P$<0.05 and **$P$<0.01.

# CHAPTER – 4

# *IN VITRO* CYTOTOXIC STUDIES OF PHENOLIC COMPOUND

# 4.1. CYTOTOXIC EFFECT OF PHENOLIC COMPOUND ON HUMAN LEUKEMIA (HL-60) CELL LINES

## Material and Methods

### Chemicals

Dimethyl sulfoxide (DMSO), Dulbecco's Modified Eagle's Medium (DMEM), Fetal bovine serum (FBS), Trypan blue, Triton X-100, Ethyl methane suphonate (EMS), Normal Melting Agarose (NMA), Low Melting Point Agarose (LMPA) and Ethidium Bromide (EtBr) were purchased from Sigma Chemicals India Ltd, ion free Phosphate Buffered Saline (PBS) was purchased from Hi-Media, India. 3,5-dinitrosalicylic acid was obtained from Merck. EMS known for mutagenic behavior was used as positive control.

### Preparation of plant extracts

The extract was obtained by infusion and maceration from 200gm of plant material. The material was weighed chopped and extracted with solvent. The infusion was prepared with 50 gm of dried leaves in 2 × 200 ml of methanol respective to its temperature and solid matter was removed by filteration. After this preliminary step, the same plant material was extracted in boiling distilled water at the same condition and the maceration was obtained following the aforementioned process at room temperature 28ºC overnight. The solvent was removed by rotary evaporation. The yield (w/w) of the infusion and maceration of methanol was 3.78 and 1.78 respectively interms of newly collected plant materials.

**Methyl Tetrazolium Cytotoxicity Assay**

The preliminary cytotoxicity analysis was done by using methyl tetrazolium (MTT). A mitochondrial enzyme in the living cells, namely succinate hydrogenase, cleaves the tetrazolium ring, converting the methyl tetrazolium (MTT) to an insoluble purple formazan. Therefore, the amount of formazan produced is directly proportional to the number of viable cells. The assay detects living, but not dead cells and signal generated is dependent on the degree of activation of the cells. This method was therefore used to measure cytotoxicity, proliferation or activation.

The colorimeter assay that measured the reduction of 3-(4-dimethylthiasol-2-yl)-2,4-diphenyltetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase was used. The MTT enters the cells and passes into the mitochondria which is reduced to an insoluble, colored, formazan product. The cells are then solubilised with an organic solvent (DMSO or isoproponal) and the released, solubilized formazan reagent is measured spectrophotometrically [82].

Human leukemia (HL-60) cells were maintained in RPMI 1640 containing 1mmol/L L-glutamine, supplemented with 10% (v/v) fetal bovine serum (FBS), 1% (w/v) penicillin/ streptomycin, and incubated at 37 ºC in humidified 5% $CO_2$ incubator. To 180 µL aliquots in six replicates of cell suspension (5× 105/mL) seeded to 96 well polystyrene tissue culture plates 20 µL aliquots of compound 4-(4-(3,4-dimethoxyphenyl)hexahydrofuro[3,4-c]furan-1-yl)-2-methoxyphenyl acetate solutions were added to each well using distilled water as solvent.

Cells incubated in culture medium alone served as a control for cell viability (untreated wells). All chemical exposures were carried in 96 well tissue culture plates for the purpose of chemical dilutions. Cells were placed in the humidified 5% $CO_2$ incubator at 37 ºC for 12,24 and 48 hours respectively. After incubation, 20 µL aliquots of MTT solution (5 mg/mL in PBS) was added to each well re-incubated for 4 hours at 37 ºC, followed by low centrifugation at 800 rpm for 5 minutes. Then, the 200 µL of supernatant culture medium was carefully aspirated and 200µL aliquots of dimethylsulfoxide (DMSO) was added to each well to dissolve the formazan crystals, followed by incubation for 10 minutes to dissolve air bubbles.

The culture plate was placed on a Biotex Model micro-plate reader and the absorbance were measured at 550 nm. The amount of colour produced is directly proportional to the number of viable cells. All assays were performed in six replicates for each concentration and means ± SD values were used to estimate the cell viability.

The cell viability rate was calculated as the percentage of MTT absorption as follows:

$$\% \text{ survival } = \frac{\text{Mean experimental absorbance}}{\text{Mean control absorbance}}$$

**Cell viability assay**

For the determination of cytotoxic activity of compound, viable cell numbers were determined using the trypan blue exclusion method [83,84]. Trypan blue dye exclusion was added to all cultures in a ratio of 1:1. Then, extract treatments and preparations were examined under the standard light microscope at 100× magnification. In principle, viable cells exclude an acid dye such as trypan blue; its uptake is indicative of irreversible membrane damage proceeding cell death. The ratio of live cells to dead cells (cell viability) was also determined. Standard curves were prepared and 50% cytotoxic concentrations of extracts ($CC_{50}$), which caused a 50% decrease in cell viability, were derived.

**Comet Assay**

The comet assay is a versatile and sensitive method for measuring single-strand and double-strand breaks in DNA [85]. Among the variety of methods developed for detecting DNA damage, comet assay or single strand cell gel electrophoresis (SSGE) assay is often used since it is fast, convenient and easy to apply. It is particularly attractive as a method to undertake *in vivo* and *in vitro* studies [86,87]. The genotoxic effect of the extracts was determined by alkaline comet assay.

The cells pellets were obtained by centrifugation (5000 rpm, 5 mins) which were again resuspended in 100 µL of Phosphate Buffered Saline (PBS). To this 100 µL of 1% low melting point agar (LMPA) was added, mixed well at 37ºC and finally layered on top of the end frosted

slides that were precoated with 1% normal melting point agarose. New cover slips (24 mm X60 mm) were placed on top of the slides and were kept at 4ºC. After 5mins the cover slips were removed and 100 µL of 0.5% LMPA was again layered on top of the slides before placing the cover slips back and keeping the slides at 4ºC again. After overnight lysis at 4 ºC in freshly prepared lysing solution (2.5 M of NaCl, 100 mM of EDTA, 10 mM of Tris and 1% Triton X-100, pH 10), slides were kept in electrophoretic unit, filled with chilled and freshly prepared electrophoresis buffer (1 mM Na2 EDTA and 300 mM NaOH, pH 13).

The slides were left in (the electrophoresis solution) for 30mins to allow unwinding of DNA. Following the unwinding, electrophoresis was performed for 30mins at 0.7V/cm using a power supply. To prevent DNA damage from stray light, all the steps starting from single cell preparation were performed under dim light. After electrophoresis, the slides were immediately neutralized with 0.4M of Tris buffer (pH 7.5) for 5mins each. The slides were then stained with EtBr (20 mg/mL:75µL pre slide) for 10mins in dark. After staining, the slides were dipped once in the chilled distilled water to remove the excess stain and subsequently, fresh cover slips were kept over them.

The slides were examined with 3-4 hours, using an image analysis system attached to fluorescent microscope. The image was transferred to a computer through a charge couple device camera and analyzed using komet 5.0 software. All the experiments were conducted in triplicates and the slides were prepared in duplicates. 25 cells per slide equaling 150 cells per group were

randomly captured at a constant depth of the gel, avoiding the cells present at the edges and superimposed comets.

**Apoptosis assay**

Apoptotic effect of the extracts at 1.25, 2.5, 5.0, 10, and 20 µg/mL concentrations was determined by Hoechst 33258 (HO) and Propidium Iodide staining, which allows for distinguishing between apoptosis and necrosis [87]. HL-60 cells ($5 \times 10^4$ per ml) were seeded in 24-well plate and treated with 1.25, 2.5, 5.0, 10, and 20 µg/mL concentrations of the extracts for 72 h. HO and PI were added directly to the culture medium to final concentrations of 5 and 2 mg/ml, respectively. After an incubation period of 1 h at 37 ºC, the cells were examined under fluorescence microscope equipped with appropriate filters for HO and PI. This method allows for a distinction between early apoptosis, late apoptosis and necrosis. Cells were counted under the microscope and the number of apoptotic cells was given as a percentage.

# RESULTS

**MTT assay of HL-60 cells lines.**

The MTT assay was used to examine the cytotoxic effect of phenolic compound 4-(4-(3,4-dimethoxyphenyl)hexahydrofuro[3,4-c]furan-1-yl)-2-methoxyphenyl acetate on HL-60 cells for 12, 24 and 48 hours, respectively. Data generated from these studies clearly indicates that exposure of the compound 4-(4-(3,4-dimethoxyphenyl)hexahydrofuro[3,4-c]furan-1-yl)-2-methoxyphenyl acetate exposure significantly reduced the viability of HL-60 cells. After 12, 24 and 48 hours of exposure, the compound exerted a significant cytotoxic effect on HL-60 cells, showing $LD_{50}$ values of 21.24 ± 0.5 µg/mL for 12 h **(Figure- 4.1.1)**, 10.25 ± 0.3 µg/mL for 24 h **(Figure- 4.1.2),** and 5.2 ± 0.6 µg/mL for 48 h **(Figure- 4.1.3)**. The overall effect of phenolic compound seems to be time dependent with regard to cell viability at different concentration **(Figure- 4.1.4)**. Similar results were observed in response to the lethal median dose ($LD_{50}$) **(Figure- 4.1.5)**.

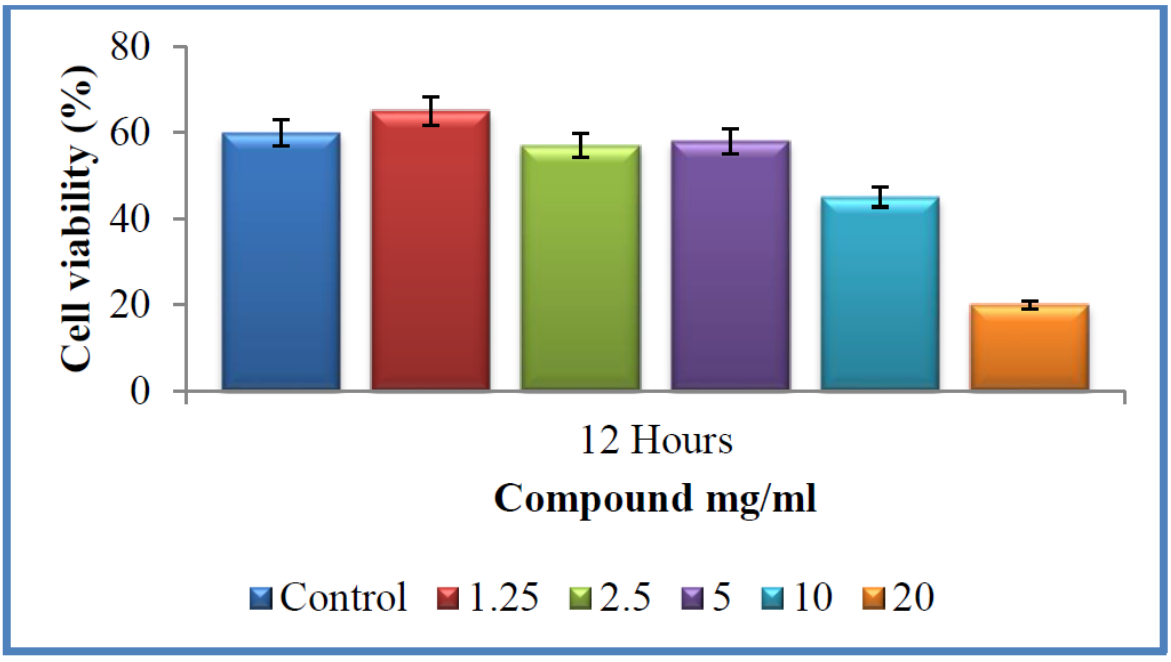



**Figure: 4.1.1**- Cytotoxic effects of phenolic compound on HL- 60 cells for 12 hours.

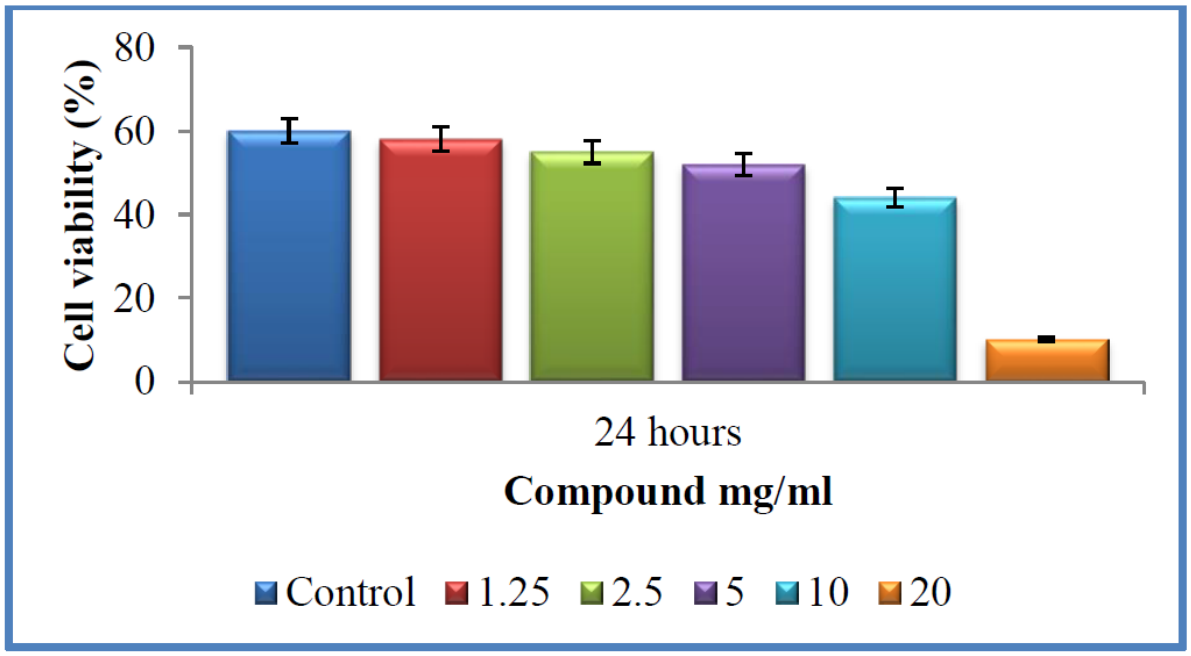



**Figure: 4.1.2**- Cytotoxic effects of phenolic compound on HL- 60 cells for 24 hours.

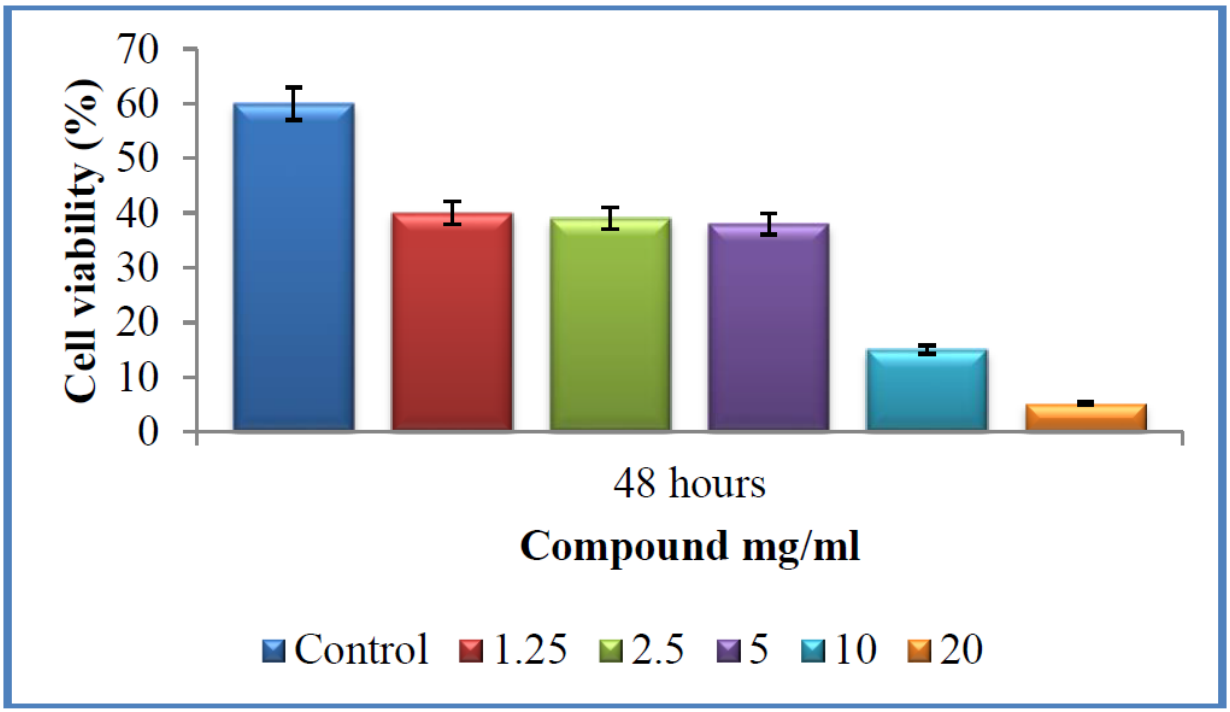



**Figure: 4.1.3**- Cytotoxic effects of phenolic compound on HL- 60 cells for 48 hours.

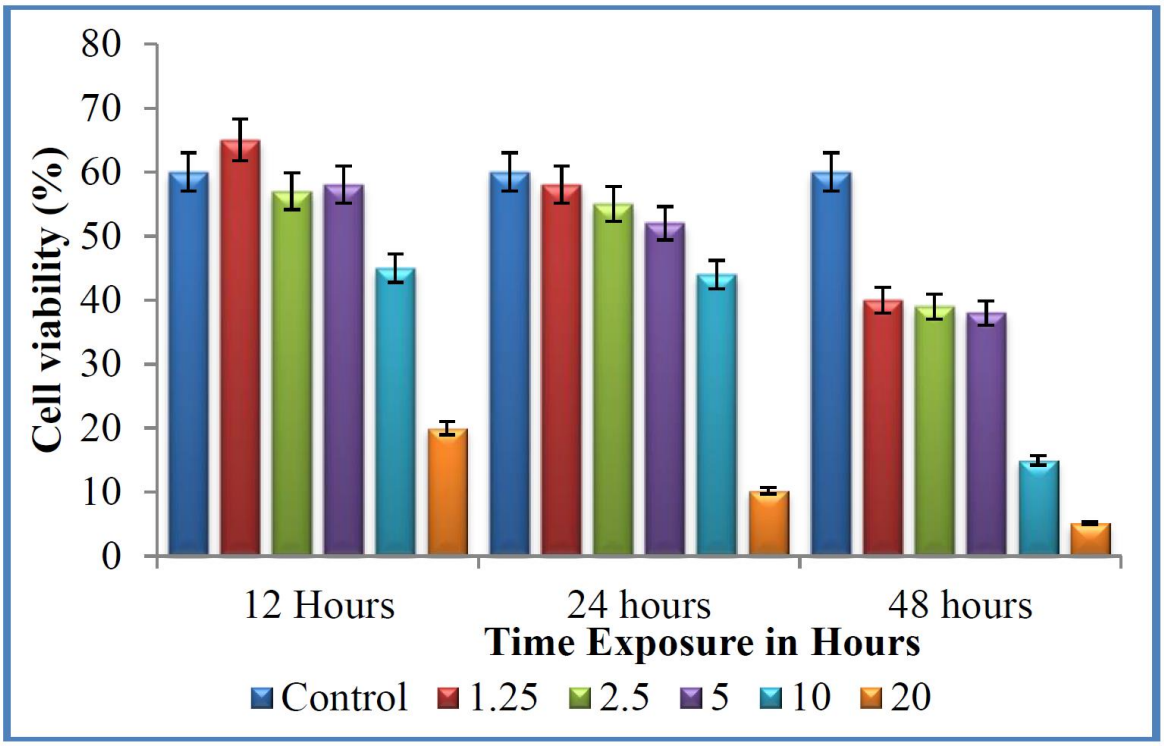



**Figure: 4.1.4**- Overall cytotoxic effects of phenolic compound on HL- 60 cells at different time interval.

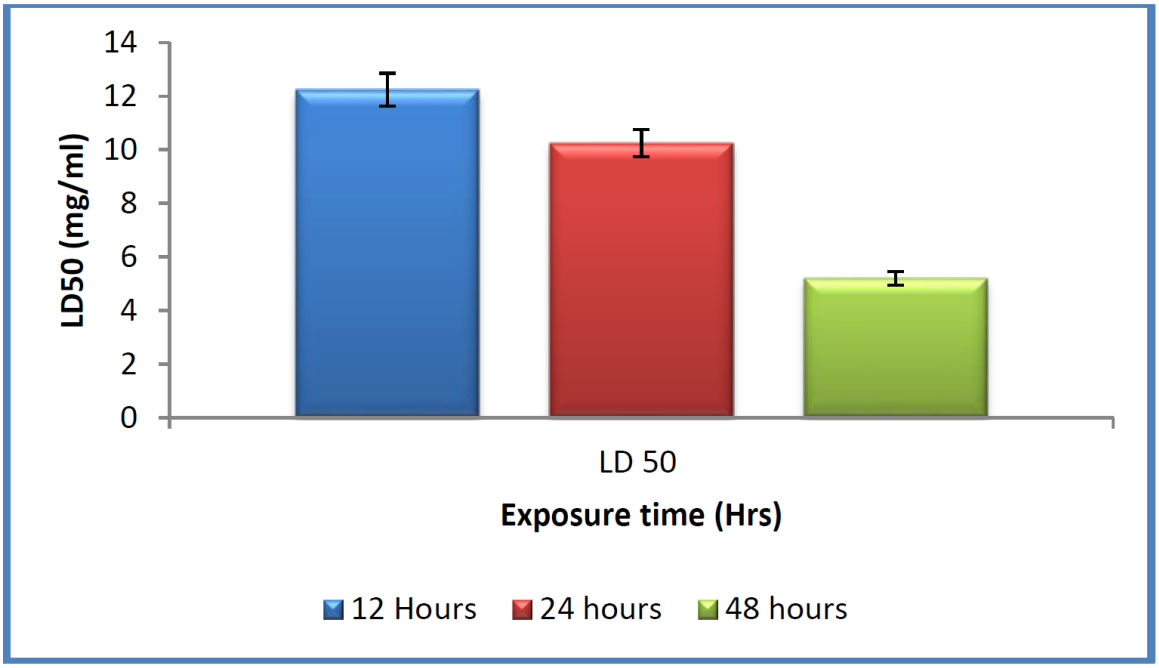



**Figure: 4.1.5**- $LD_{50}$ values in response to time exposure

**Comet Assay**

Cytotoxicity assessment is way to find out the immediate response of cells, which come in contact with a foreign particle where as the genotoxicity of those reveals a long term response. Thus it is very important to investigate the genotoxicity of those concentrations that reveals the cytotoxicity. The percentage DNA pattern of HL-60 cells treated with different concentrations of isolated compound. The % DNA value of control cells was recorded to be 5.05 units. There was a very slight increase in the samples treated with particles showing values of % DNA at 5.58%, 5.43, 5.77 and 5.97 (Image- a, b, c and d) corresponding to 1.25 µg/mL, 2.50 µg/mL, 5.0 µg/mL, 10.0 µg/mL respectively of phenolic compound. The resultant comet pattern of the HL-60 cells also showed an intact head and complete absence of DNA fragments in the form of tail, suggesting that the doses were not genototoxic. The metabolic compound at 20 µg/ml concentration showed increase in % DNA of 6.21 units (Image- 4.1.1 e) when compared to control 5.35. This increase in the % DNA pattern was also found non significant when analyzed with ANOVA.

**Apoptotic Effects**

HL-60 cells were treated with various doses [1.25(a), 2.5(b), 5.0(c), 10(d), and 20(e) µg/mL] of phenolic compound for 72 h. As indicated in Table- 3.2.1, apoptosis induction was moderate (8.66 to 22.00%) after treating with DMSO, while the test sample showed significantly high apoptotic induction after 72 h and this effect was dose-dependent, these values were found tobe statistically significant. Apoptotic effect of extracts on HL-60 cells after 72 h was found to be increased in methanol. Furthermore, it was found that the test sample induced slight necrosis in HL-60 cells.The change of nuclear morphology in the HL-60 cells after treatment with 1.25, 2.5, 5.0, 10, and 20 µg/mL extracts for 72 h was observed under fluorescence microscopy

(Image- 4.1. 2). Ho and PI staining showed that there were significant morphological changes in the nuclear chromatin. In the untreated group, the cells were stained with a less bright blue color which was homogeneous.

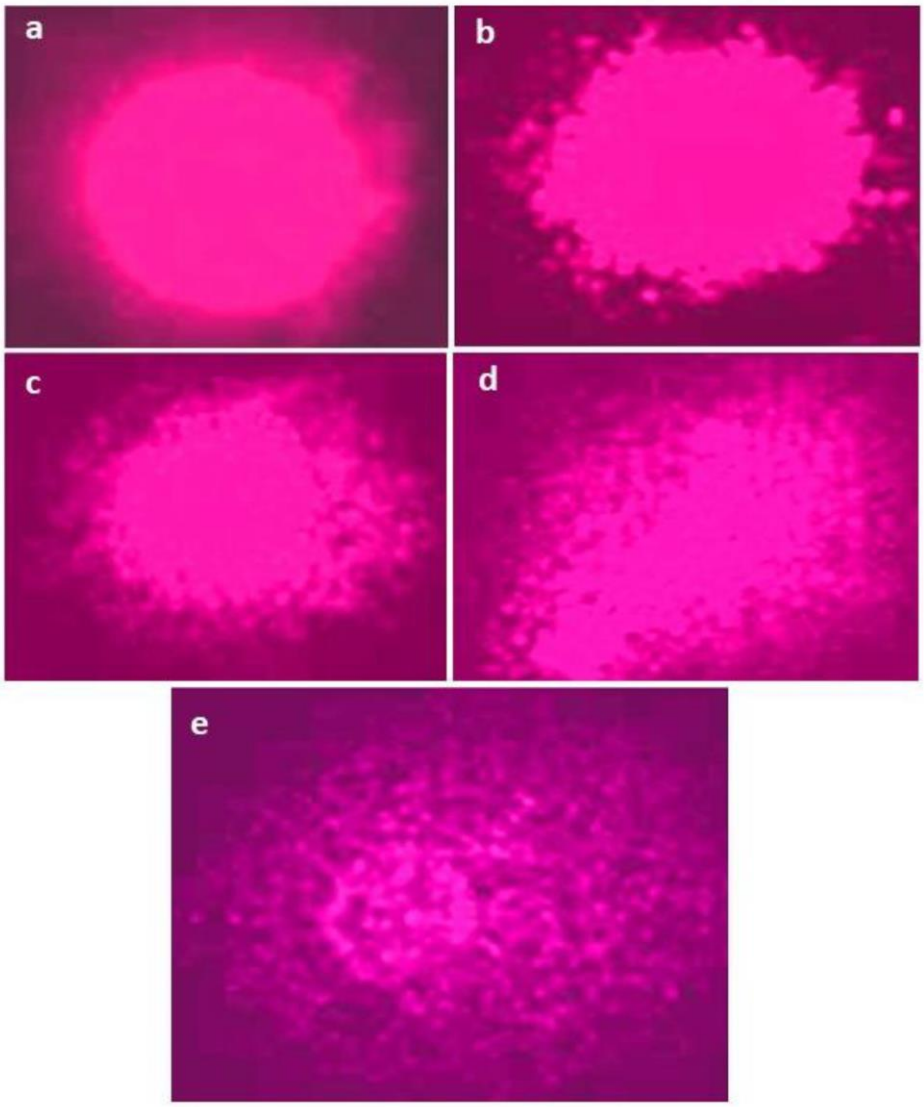


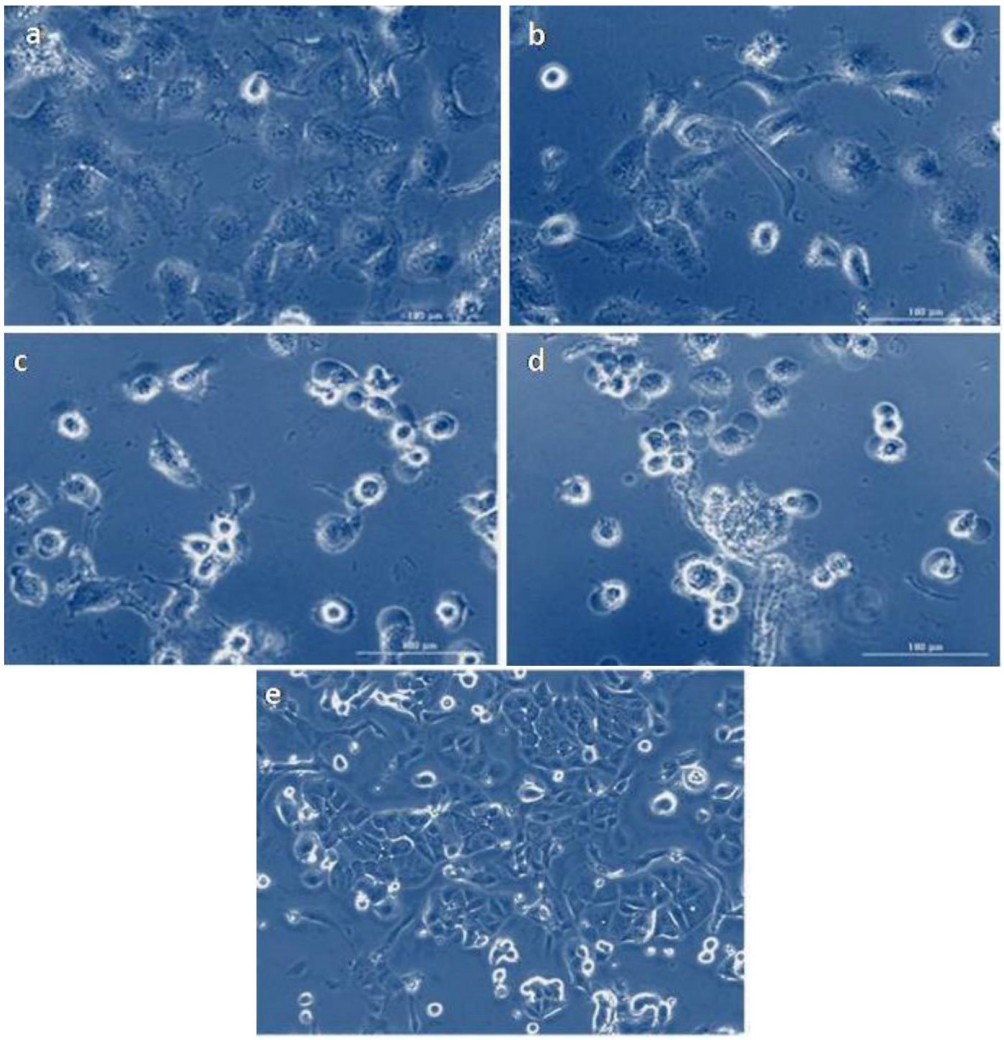

**Image: 4.1.1**- Cytotoxic effect of phenolic compound showing % of DNA damage at 1.25(a), 2.50(b), 5.0(c), 10.0(d), 20.0 μg/ml (e) in HL-60

After treating with phenolic compound for 72 h, the blue emission light in apoptotic cells was much found to be much brighter than the control cells while necrotic cells appeared in violet-red fluorescence without chromatin condensation and apoptotic bodies. As shown in Table, all of the cells treated by extract showed typical apoptotic features in the treated cells, including cell shrinkage, nuclear fragmentation marginalization, chromatin condensation and production of membrane-bound apoptotic bodies. Apoptotic bodies, a specific and distinct feature of apoptotic cells, were found in the majority of treated cells. These results suggest that their inhibitory effect on cell growth might be through induction of apoptosis.

**Table: 4.1.1 -** Apoptotic effects of the *Pandanus odorattissimus* L compound on HL-60 cells.

| Sample | Concentration (µg/ml) | Total cells | Apoptotic cells (% ± SD) | Necrotic cell (% ± SD) |
|---|---|---|---|---|
| | | 200 | 1.66±0.00 | -- |
| Control | 0.1% | 200 | 2.00±0.01 | -- |
| Solvent | 1.25 | 200 | 9.56 ± 0.21* | 1.00 ± 0.00 |
| | 2.50 | 200 | 10.00 ± 0.47* | 2,03 ± 0.31 |
| | 5.00 | 200 | 11.36 ± 0.60* | 3.01 ± 0.91 |
| | 10.00 | 200 | 12.82±0.26 | 3.98 ± 0.26 |
| | 20.00 | 200 | 14.17±0.43 | 4.85± 0.36 |
| Compound | 1.25 | 200 | 29.00 ± 0.60* | 7.00 ± 0.05 |
| | 2.50 | 200 | 38.33 ± 0.58* | 9.33 ± 0.06* |
| | 5.00 | 200 | 47.35 ± 1.00* | 11.00 ± 0.09* |
| | 10.00 | 200 | 51.23 ± 0.50* | 12,46 ± 0.90* |
| | 20.00 | 200 | 56.44±0.25* | 13. 56± 0.15* |

* P values ($p<0.05$) was found to be statically significant.




**Imag : 4.1.2 -** Apoptotic effect of phenolic compound in HL-60 cell lines at 1.25(a), 2.5(b), 5.0(c), 10(d) and 20(e) µg/mL.

## 4.2. CYTOTOXIC EFFECT OF PHENOLIC COMPOUND ON MDA-MB-231

### Material and Methods

### Chemicals

Dimethyl sulfoxide (DMSO), Dulbecco's Modified Eagle's Medium (DMEM), Fetal bovine serum (FBS), Trypan blue, Triton X-100, Ethyl methane suphonate (EMS), Normal Melting Agarose (NMA), Low Melting Point Agarose (LMPA) and Ethidium Bromide (EtBr) were purchased from Sigma Chemicals India Ltd, ion free Phosphate Buffered Saline (PBS) was purchased from Hi-Media, India. 3,5-dinitrosalicylic acid was obtained from Merck. EMS known for mutagenic behavior was used as positive control.

### Preparation of plant extracts

The extract was obtained by infusion and maceration from 200g of plant material. The material was weighed chopped and extracted with solvent. The infusion was prepared with 50 gm of dried leaves in 2 × 200 ml of methanol respective to its temperature and solid matters were removed by filtration. After this preliminary step, the same plant material was extracted in boiling distilled water at the same condition and the maceration was done following the aforementioned process at room temperature 28ºC overnight. The solvent was removed by rotary evaporation. The yield (w/w) of the infusion and maceration of methanol was 3.78 and 1.78 respectively in terms of newly collected plant material.

**Cytotoxicity assay**

The preliminary cytotoxicity analysis was done by using methyl tetrazolium (MTT). A mitochondrial enzyme in the living cells, namely succinate hydrogenase was used, cleaves the tetrazolium ring, converting the methyl tetrazolium (MTT) to an insoluble purple formazan. Therefore, the amount of formazan produced is directly proportional to the number of viable cells. The assay detects living, but not dead cells and signal generated is dependent on the degree of activation of the cells. This method was therefore used to measure cytotoxicity, proliferation or activation.

The colorimeter assay that measures the reduction of 3-(4-dimethylthiasol-2-yl)-2,4-diphenyltetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. The MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, colored, formazan product. The cells are then solubilised with an organic solvent (DMSO or isoproponal) and released solubilized formazan reagent is measured spectrophotometrically [87,88].

MDA-MB-231 cells were maintained in RPMI 1640 containing 1 mmol/L L-glutamine, supplemented with 10% (v/v) fetal bovine serum (FBS), 1% (w/v) penicillin/ Streptomycin and incubated at 37 ºC in humidified 5% $CO_2$ incubator. To 180 µL aliquots in six replicates of cell suspension (5× 105/mL) seeded to 96 well polystyrene tissue culture plates, 15µL aliquots of compound 4-(4-(3,4-dimethoxyphenyl) hexahydrofuro [3,4-c]furan-1-yl)-2-methoxyphenyl acetate solutions (1.25, 2.5, 5.0, 10, and 20 µg/mL) were added to each well using distilled water as solvent.

Cells incubated in culture medium alone served as a control for cell viability (untreated wells). All chemical exposures were carried in 96 well tissue culture plates for the purpose of chemical dilutions. Cells were placed in the humidified 5% $CO_2$ incubator at 37 ºC for 24, 48 and 72 hours respectively. After incubation 20 µL aliquots of MTT solution (5 mg/mL in PBS) were added to each well re-incubated for 4 hour at 37ºC, followed by low centrifugation at 800 rpm for 5 minutes. Then, the 200 µL of supernatant culture medium was carefully aspirated and 200 µL aliquots of dimethylsulfoxide (DMSO) were added to each well to dissolve the formazan crystals followed by incubation for 10 minutes to dissolve air bubbles.

The culture plate was placed on a Biotex Model micro-plate reader and the absorbance was measured at 550 nm. The amount of colour produced is directly proportional to the number of viable cells. All assays were performed in six replicates for each concentration and means ±SD values were used to estimate the cell viability.

**Cell viability assay**

For the determination of cytotoxic activity of compound, viable cell numbers were determined using the trypan blue exclusion method [88]. Trypan blue dye exclusion was added to all cultures in a ratio of 1:1. The extract treatments and preparations were examined under the standard light microscope at 100× magnification. In principle, viable cells exclude an acid dye such as trypan blue; its uptake is indicative of irreversible membrane damage proceeding cell death. The ratio of live cells to dead cells (cell viability) was also determined. Standard curves were prepared and 50% cytotoxic concentrations of extracts ($CC_{50}$) which caused a 50% decrease in cell viability were derived.

**Determination of DNA damaging effects of the extracts (Alkaline comet assay)**

The comet assay is a versatile and sensitive method for measuring single-strand and double-strand breaks in DNA [89]. Among the variety of methods developed for detecting DNA damage, comet assay or single cell gel electrophoresis (SSGE) assay is often used since it is fast, convenient and easy to apply. It is particularly attractive as a method to undertake *in vivo* and *in vitro* studies [90,91]. The genotoxic effect of the extracts was determined by alkaline comet assay.

The cell pellets were obtained by centrifugation (5000 rpm, 5 mins) which were again resuspended in 100 µL of PBS. To this 100 µL of 1% LMPA (low melting point agar) was added, mixed well at 37 ºC and finally layered on top of the end frosted slides that were precoated with 1% normal melting point agarose. Now cover slips (24 mm X60 mm) were placed on top of the slides and were kept at 4 ºC. After 5 mins the cover slips were removed and 100 µL of 0.5% LMPA was again layered on top of the slides before placing the cover slips back and keeping the slides at 4ºC again. After overnight lysis at 4ºC in freshly prepared lysing solution (2.5 M of NaCl, 100 mM of EDTA, 10 mM of Tris and 1% Triton X-100, pH 10) slides were kept in electrophoretic unit, filled with chilled and freshly prepared electrophoresis buffer (1 mM Na2EDTA and 300 mM NaOH, pH 13).

The slides were left in (the electrophoresis solution) for 30 mins to allow unwinding of DNA. Following the unwinding, electrophoresis was performed for 30 mins at 0.7V/cm using a power supply. To prevent DNA damage from stray light, all the steps starting from single cell preparation were performed under dim light. After electrophoresis, the slides were immediately

neutralized with 0.4M of Tris buffer (pH 7.5) for 5 mins each. The slides were then stained with EtBr (20 mg/mL:75 µL pre slide) for 10 mins in dark. After staining, the slides were dipped once in the chilled distilled water to remove the excess stain and subsequently, fresh cover slips were kept over them.

The slides were examined with 3-4 hours, using an image analysis system attached to fluorescent microscope. The image was transferred to a computer through a charge couple device camera and analyzed using komet 5.0 software. All the experiments were conducted in triplicates and the slides were prepared in duplicates. 25 cells per slide equaling 150 cells per group were randomly captured at a constant depth of the gel, avoiding the cells present at the edges and superimposed comets.

## RESULTS

### MTT Cytotoxic Assay

The MTT assay was used to examine the cytotoxic effect of phenolic compound 4-(4-(3,4-dimethoxyphenyl)hexahydrofuro[3,4-c]furan-1-yl)-2-methoxyphenyl acetate on MDA-MB-231 cells for 24, 48 and 72 hours, respectively. Data generated from these studies clearly indicates that exposure of the compound to MDA-MB-231 significantly reduced the viability of HL-60 cells. After 24, 48 and 72 hours of exposure, the compound exerted a significant cytotoxic effect on MDA-MB-231 cells, showing $LD_{50}$ values of 12.24 ± 0.5 µg/mL for 24 h **(Fig. 4.2.1)**, 10.25 ± 0.3 µg/mL for 48 h **(Fig. 4.2.2),** and 5.2 ± 0.6 µg/mL for 72 h **(Fig. 4.2.3)**. The effect of phenolic compound seems to be time dependent with regard to cell viability in different doses of compound it was compared with control **(Fig. 4.2.4)**.

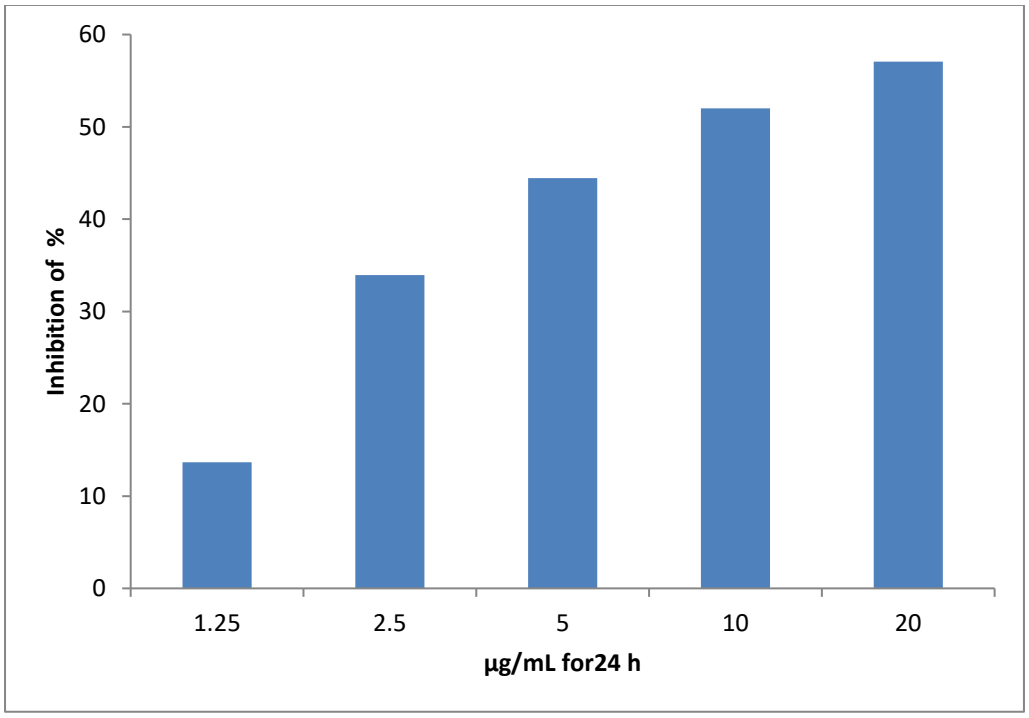



**Fig. 4.2.1:** The cytotoxic effect of phenolic compound 4-(4-(3,4-dimethoxyphenyl)hexahydrofuro[3,4-c]furan-1-yl)-2-methoxyphenyl acetate on MDA-MB-231 cells for 24 hours.

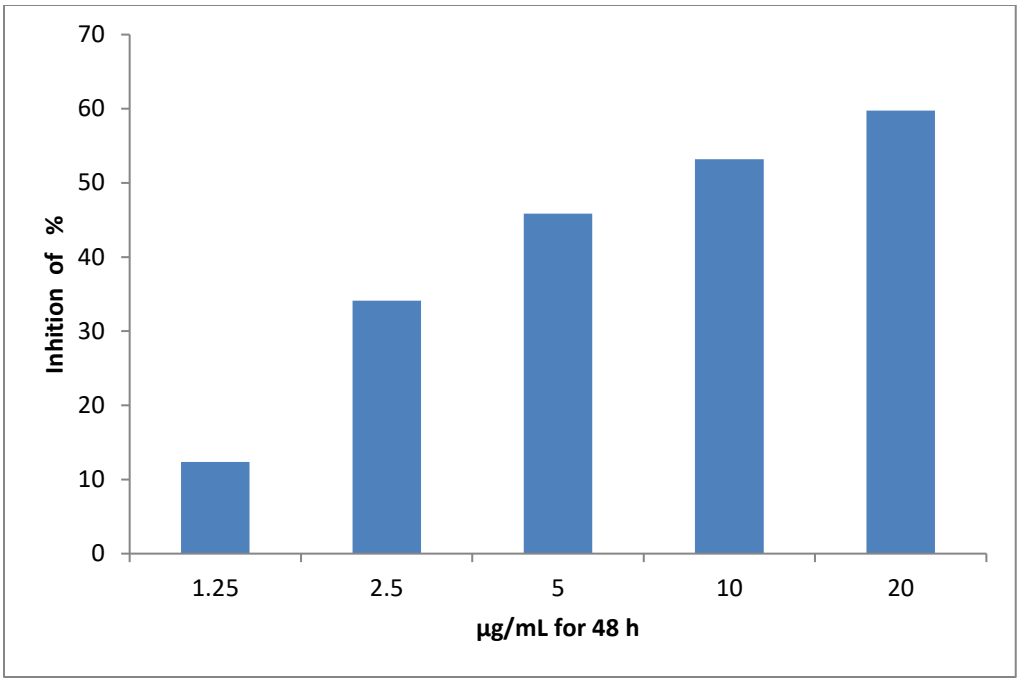



**Fig. 4.2.2:** The cytotoxic effect of phenolic compound 4-(4-(3,4-dimethoxyphenyl)hexahydrofuro[3,4-c]furan-1-yl)-2-methoxyphenyl acetate on MDA-MB-231 cells for 48hours.

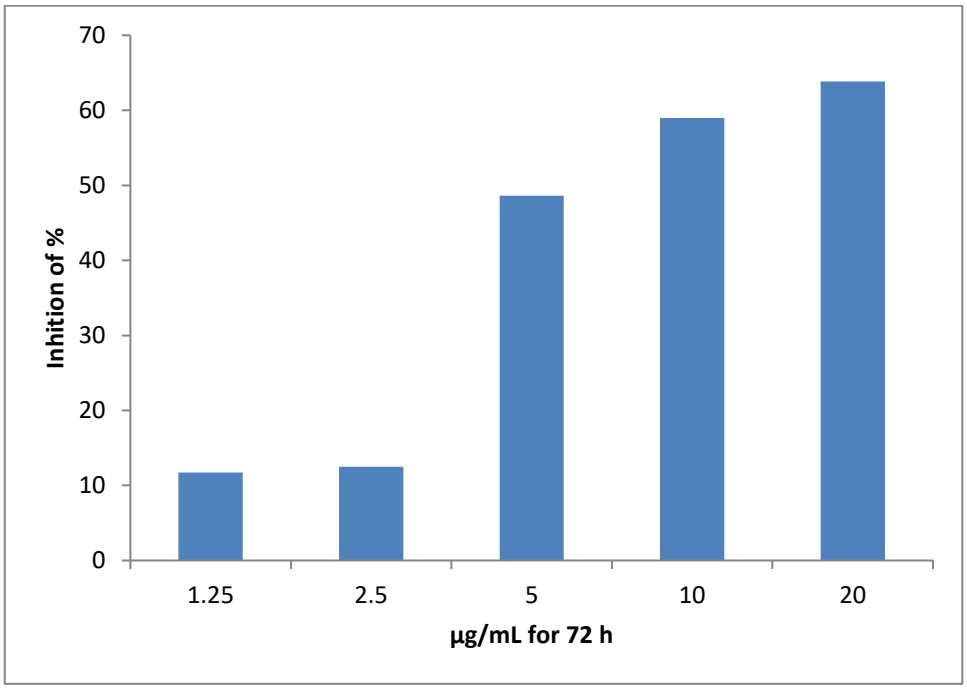



**Fig. 4.2.3:** The cytotoxic effect of phenolic compound 4-(4-(3,4-dimethoxyphenyl)hexahydrofuro[3,4-c]furan-1-yl)-2-methoxyphenyl acetate on MDA-MB-231 cells for 72 hours

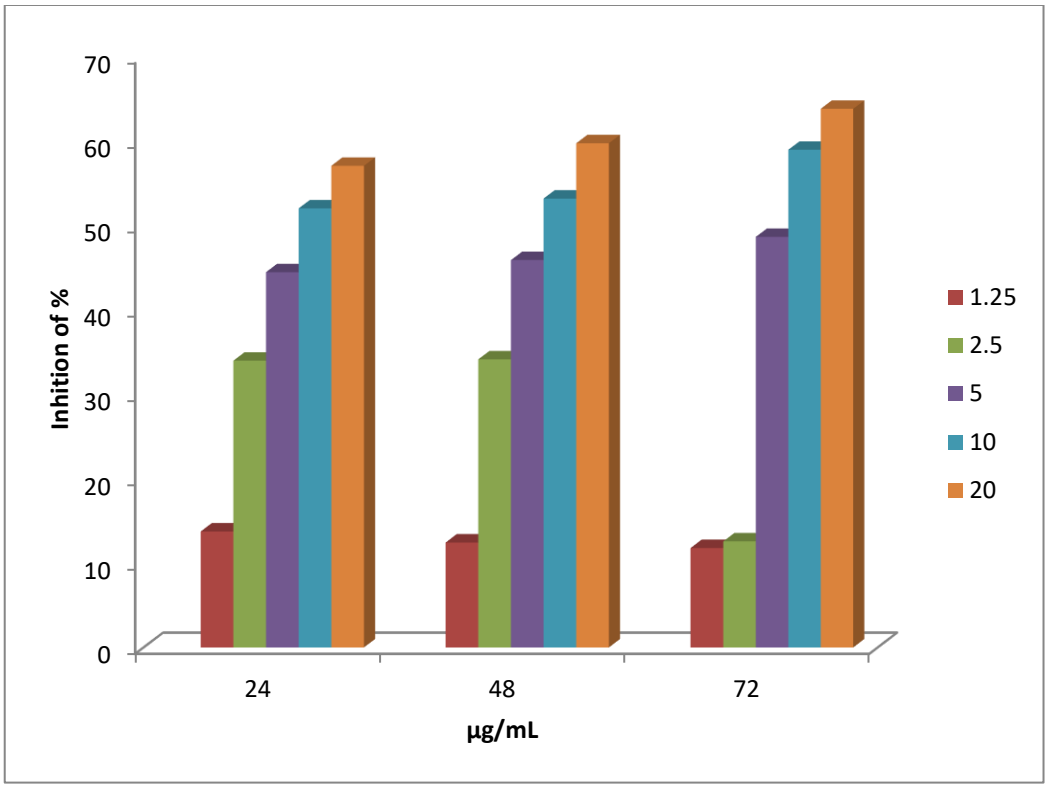



**Fig. 4.2.4:** The cytotoxic effect of phenolic compound 4-(4-(3,4-dimethoxyphenyl)hexahydrofuro[3,4-c]furan-1-yl)-2-methoxyphenyl acetate on MDA-MB-231 cells for 24, 48 and 72 hours

**Comet Assay**

Cytotoxicity assessment is way to find out the immediate response of cells, which come in contact with a foreign particle where as the genotoxicity of those reveals, a long term response. Thus it is very important to investigate the genotoxicity of those concentrations that reveals the cytotoxicity. The Image **4.2.1** the percentage DNA damage pattern of MDA-MB-231 cells treated with different concentrations of isolated compound. The percentage of DNA value of control cells was recorded to be 5.05 units. There was a very slight increase in the samples treated with particles with percentage of DNA at 5.20, 5.35% units, 5.42 units, 5.68 units and 5.87 units corresponding to 1.25 µg/mL, 2.50 µg/mL, 5.0 µg/mL, 10.0 µg/mL and 20.0 µg/mL

respectively of phenolic compound. The resultant comet pattern of the MDA-MB-231 cells also showed an intact head and complete absence of DNA fragments in the form of tail **(Image 4.2.1)**, suggesting that the doses were not genototoxic. The metabolic compound at 20 µg/ml, concentration showed increase in percentage tail DNA 6.01 units when compared to control. This increase in percentage of tail DNA pattern was found non significant when analyzed with ANOVA.

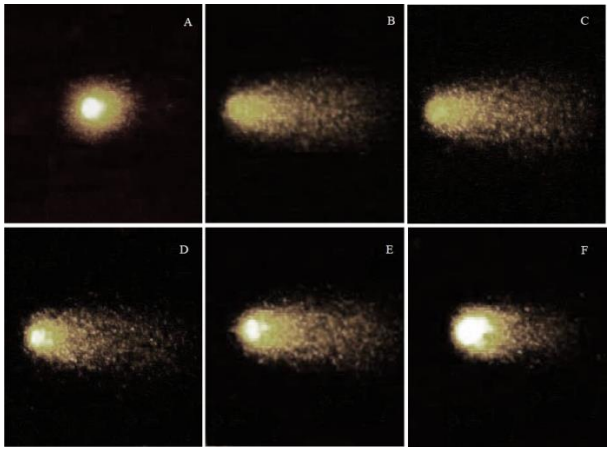



**Image 4.2.1**: Cytotoxic effect of phenolic compound showing % of DNA damage at A = control, B =1.25 µg/mL, C = 2.50 µg/mL, D = 5.0 µg/mL, E = 10.0 µg/mL and F = 20.0 µg/mL respectively in MDA MB – 231.

## 4.3. MDA- MB-468 (BASAL-A MAMMARY CARCINOMA) ACTIVITY OF PHENOLIC COMPOUND

### Materials and Methods

### Chemicals

Dimethylsulfoxide (DMSO), Dulbecco's Modified Eagle's Medium (DMEM), Fetal bovine serum (FBS), Trypan blue, Triton X-100, Ethyl methane suphonate (EMS), Normal Melting Agarose (NMA), Low Melting Point Agarose (LMPA) and Ethidium Bromide (EtBr) were purchased from Sigma Chemicals India Ltd. Ion free Phosphate Buffered Saline (PBS) was purchased from Hi-Media, India. 3,5-,dinitrosalicylic acid was obtained from Merck. EMS known for mutagenic behaviour was used as positive control.

### Preparation of plant extracts

The extract was obtained by infusion and maceration from 200 g of plant material. The material was weighed chopped and extracted with solvent. The infusion was prepared with 50 gm of dried leaves in 2 X 200 ml of methanol respective to its temperature and solid matter was removed by filtration. After this preliminary step, the same plant material was extracted in boiling distilled water under the same condition and the maceration was obtained following the aforementioned process at room temperature 28□C overnight. The solvent was removed by rotary evaporation. The yield (w/w) of the infusion and maceration of methanol was 3.78 and 1.78, respectively in terms of newly collected plant materials.

**Preparation of stock solution**

The dried seed extract was dissolved in 1 ml of DMSO (0.1% v/v) and made up to 10 ml with complete media (MEM) to give stock solution of compound 10 mg/ml.

**Extract dilution**

Stock solution was diluted with complete media to obtain the concentrations of 5, 10 and 20 µg/ml. All were stored in air tight container until tested.

**Cell culture**

MDA-MB-468 (basal-A mammary carcinoma) breast cancer cell lines and L929 normal fibroblast cell lines were obtained from NCCS, Pune. MDA-MB-468 breast cancer cell lines and L929 normal cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM), while L929 normal cells were cultured in Roswell Park Memorial Institute 1640 (RPMI-1640) medium supplemented with 10% v/v foetal bovine serum (FBS), 100 U/mL of penicillin, and 100 g/mL of streptomycin as a complete growth medium. Cells were maintained in 25cm2 flasks and incubated in a humidified incubator ($CO_2$Water-Jacketed Incubator NuAire, Fernbrook Lane, Plymouth, USA) at $37^0$C with 5% $CO_2$.

**Cytotoxicity analysis by MTT assay**

The preliminary cytotoxicity analysis was done using methyl tetrazolium (MTT). A mitochondrial enzyme in the living cells, namely succinate hydrogenase, cleaves the tetrazolium ring, converting the methyl tetrazolium (MTT) to an insoluble purple formazan. Therefore, the amount of formazan produced is directly proportional to the number of viable cells. The assay detects living, but not dead cells and signal generated is dependent on the degree of activation of the cells. This method was therefore used to measure cytotoxicity, proliferation or activation [92].

Cytotoxicity testing was performed using 3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma). In this assay, cells were harvested after reaching 80% confluence. Before starting the MTT assay, cells were optimised at different seeding densities ranging from $2.0 \times 10^3$ to $1.0 \times 10^6$ cell/mL in light to determine appropriate seeding number for the experiment. Each well of the microtiter plate (96-well) was filled with 100µL of cell suspension MDA-MB-468 in complete growth medium. After 24 h of incubation, cells were treated with compound of different concentrations ranging from 5 to 20 µg/mL, with a total well volume of 200µL with technical replicates. Microtiter plates were further incubated for 72 h with plant extracts. After 72 h of incubation, 20µL of MTT (a stock solution of 5mg/mL in PBS) was added to each well and the plates incubated for 4 h at $37^0$C. Medium from each well was carefully removed without disturbing the MTT crystals in wells. The MTT formazan crystals were dissolved by the addition of 1M HCl and 100mM isopropanol to each well. After solubilizing the purple formazan, absorbance was measured at a wavelength of 575 nm:

$$\% \text{ survival } = \frac{\text{Mean experimental absorbance}}{\text{Mean control absorbance}}$$

**DNA fragmentation**

The cells were incubated with an $IC_{50}$ concentration of 20 µg/ml for 5 days. The treated cells were collected every day by trypsinization. DNA was extracted once, with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and once with chloroform:isoamyl alcohol (24:1). The DNA was precipitated with a two-third volume of cold isopropanol followed by centrifugation. The DNA pellet was washed once in 70% ethanol and resuspended in deionized water containing 0.1 mg/mL. DNA was analyzed by 1.5% agarose gel electrophoresis [93].

**Statistical analysis**

Student's *t*-test was used to analyze intergroup differences. Experiments were repeated at least three times, and data was represented as the mean ± SD. A *p*-value of less than 0.05 was considered to be statistically significant.

# RESULTS

## Determination of $IC_{50}$ concentration using MTT assay

The $IC_{50}$ concentration of phenolic compound of *Pandaus odorattissimus* L. in MDB-MB-468 cells was determined by MTT assay. The inhibition activity of MDB-MB-468 cells at different concentration such as 5, 10 and 20 µg/ml of test compound is depicted **Figure 4.3.1.** The $IC_{50}$ value was determined based on cell viability rates. 5.79±0.09 inhibition was observed in concentration of 20 µg/ml which has been taken as $IC_{50}$ value and fixed for our further study

## The DNA fragmentation of 20 µg/ml on breast cancer cell lines

A DNA fragmentation assay was used to determine whether the action of 20 µg/ml concentration of compound was associated with apoptosis or not. In this experiment, all tested cell lines were incubated with 20 µg/ml at the $IC_{50}$ concentration. DNA fragmentation was found in MDA-MB-468 and L929 cells at 1,2, 3, 4 and 5 days after exposure. Therefore, the cytotoxic effect of 20 µg/ml was selected for breast cancer cell lines and mediated through the induction of apoptosis.

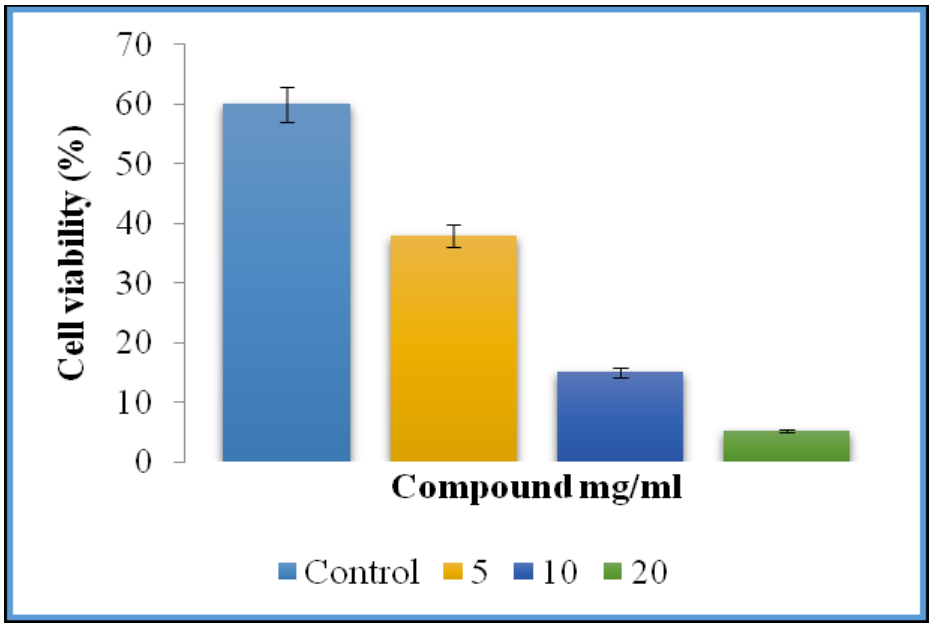



**Figure 4.3.1:** Dose dependent Cytotoxicity effect of Phenolic compound over cell viability on MDA MB- 468 cells.

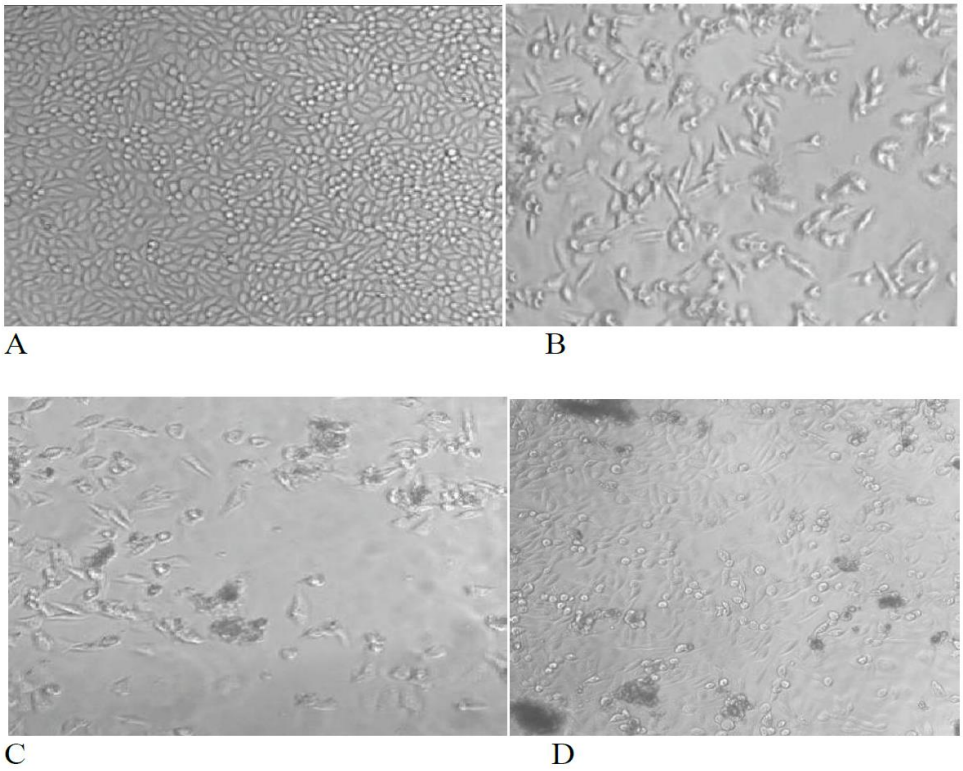
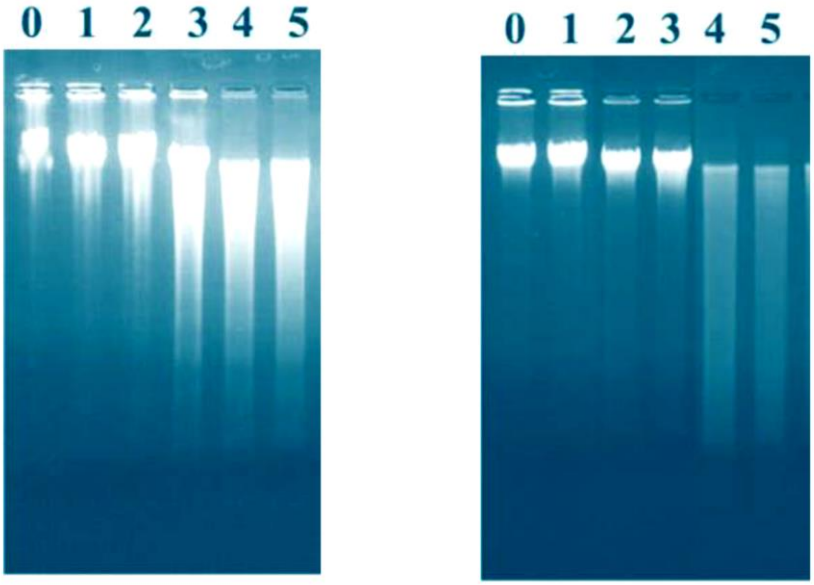



**Image -4.3.1:** Phase contrast image of MDB-MB-468 cell lines at 10X magnification. (A) Normal MDB-B-468 cells (B) 5 µg/ml (C) 10 µg/ml (D) 20 µg/ml.A.




**(A) MDA-MB-468** **(B) L929**

**Image.-4.3.2:** DNA fragmentation induced by phenolic compound (20µg/ml) compound in two cell lines. Cells were treated for five days with 20µg/ml, and DNA fragmentation was assessed by 1.5% agarose gel electrophoresis and ethidium bromide staining. 20µg/ml was used to treat (**A**) MDA-MB-468 and (**B**) L929 with 2.69 µg/mL, 2.97 µg/mL, 3.98µg/mL, respectively. The data are representative of three independent experiments carried out under the same conditions.

## 4.4. CYTOTOXIC EFFECT OF PHENOLIC COMPOUND ON KB CELLS (ORAL CANCER)

### Preparation of extracts and stock solutions

The extract was obtained by infusion and maceration from 200 g of plant material. The material was weighed chopped and extracted with solvent. The infusion was prepared with 50 gm of dried leaves in 2 ml× 200 ml of methanol respective to its temperature and solid matters were removed by filtration. After this preliminary step, the same plant material was extracted in boiling distilled water at the same condition and the maceration was done following the aforementioned process at room temperature 28°C overnight. The solvent was removed by rotary evaporation. The yield (w/w) of the infusion and maceration of methanol was 3.78 and 1.78 respectively in terms of newly collected plant material. Dimethyl sulfoxide (DMSO) was used to dissolve each extracts and sterilized using 0.22 μm syringe filters (Axiva, Scichem Biotech)for further use.

### Antiproliferative assay

A KB (oral cancer) cell line for oral cancer was procured from National Centre for Cell Science (NCCS) Pune, used to study antiproliferative activity. Cells were grown in DMEM-F12 (Sigma, Germany), having 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin solution. Cells were incubated in $CO_2$ incubator containing 5% $CO_2$at 37°C temperature.

**Maintenance of KB cell lines**

Cell culture flasks were selected by the method of confluency. They were observed under inverted microscope. In order to maintain cell line, the technique of sub culturing was performed. This is to facilitate cell growth by extraction of cells from the existing medium and placing them into a fresh new medium. For cell maintenance, enzymatic methods using TPVG were revalently used. Growth medium was then extracted completely from the flasks and further subjected to incubation at 37°C after the addition of enzyme. This, initially, detach the cells from the surface.

**Cytotoxic activity**

In MTT assay, cytotoxicity of methanol extracts of all selected plant extracts were determined on KB (oral cancer) cells depended on the reduction of MTT dye (Sigma, Germany) by calculating the quantity of insoluble formazan produced in liver cells. Briefly, in96-well plate the cells were inoculated in 100 µl at number varying from 10,000 to 20,000 cells/well based on cell growth characteristics. Then the 96-well plates were kept at 37°C and 5% $CO_2$ humidified incubator for 24 h before addition of plant extracts. Furthermore, methanol extracts dissolved in 2% DMSO were added into a 96-wellplate at different concentration (25, 50, 100, 200 µg/ml) by diluting it in medium. After 24 h, in each well 20 µl of MTT reagent (5 mg/ml) was added. After 4 h of incubation 100 µl DMSO was added, this was followed by 1 h of incubation at 37°C to

dissolve formazan crystals. Using an ELISA plate reader (Promega, USA) absorbance was taken at 570 nm and background wavelength at 750 nm [94].

Following equation was used to calculate the percentage of cell viability: Cell viability (%) = (OD sample (mean)/ (OD control (mean) × 100, where, OD is the optical density; OD of sample: Mean absorbance of treated cells and OD of control: Mean absorbance of control cells. Treatment of KB cells with *Pandanus odorattissimus* L extract: Aliquot of the extract (25 micro grams and 50 micro grams) was added and incubated with KB cell lines.

**Isolation of DNA**

$1 \times 10^6$ cells were incubated with 100 µl of cell lysis buffer at room temperature for one hour. This was centrifuged for 15 min at 3000 rpm at 4°C to sediment the cell debris. To the supernatant equal volume of phenol: chloroform: isoamyl alcohol mixture was added to the supernatant and mixed well. This was centrifuged at 5000 rpm for 15 min. The supernatant was transferred to new tube. The 3rd step was repeated once. To the final aqueous phase 40 µl of 3.5 M ammonium acetate was added, to this ice cold isopropanol was added to precipitate the DNA. This was incubated at -20°C for 1hour, followed by the centrifugation at 10000 rpm for15 min. The pellet was retained and washed with 70% ethanol and stored in 20 µl to 50 µl of TE buffer. The samples were analyzed in 2%agarose gel stained with Ethidium bromide.

**DNA fragmentation by agarose gel electrophoresis**

The extracted DNA was loaded to agarose gel with the loading dye, DNA fragments were visualized under UV transilluminator. DNA fragmentation was observed with all the two concentrations of *Pandanus odorattissmus* L extract on oral cancer cell lines by agarose gel electrophoresis. Apoptosis is characterized biochemically by the activation of a nuclear endonuclease that cleaves the DNA into multimers of 180 to 200 base pairs and can be visualized as an 'oligosomal ladder' by standard agarose gel electrophoresis. This proves that *Pandanus odoratissmus* L. extract shows apoptotic activity on the oral cancer cells by degrading its DNA [95].

# RESULTS

## Activity of KB cell line

Cytotoxicity study was performed using *Pandanus odorattissimus* L methanol extracts on KB cell line. In some cases, dose-dependent inhibition of KB cell growth occurred, especially when the cells were treated at higher concentrations. Percentage of inhibition observed at 12.5 µg/mL of the test compound was (32%, 36% and 39%) at 25 µg/mL (42%, 45% and 46%) 50 µg/ mL (45%, 50% and 55%) and 100µg/mL (57%, 61% and 62%) after 24, 48 and 72 h of treatments (Figure- 4.4.1). The $IC_{50}$ values of 100 µg/mL, 50 µg/mL and 25 µg/mL were determined for 24, 48 and 72 h treatment, which indicates that the extract is only highly cytotoxic to KB cells after 72 h treatment.

## DNA Fragmentation Analysis

Induction of apoptosis on KB cells by *Pandanus odorattissmus* L. extract was validated by DNA fragmentation analysis using gel electrophoresis. The DNA bands obtained from both extract-treated KB produced ladder pattern as observed from Lane 1to 4 (Image- 4.4.1). A ladder formation was used to indicate that the DNA has undergone fragmentation, and each fragment corresponded to a band in the ladder.

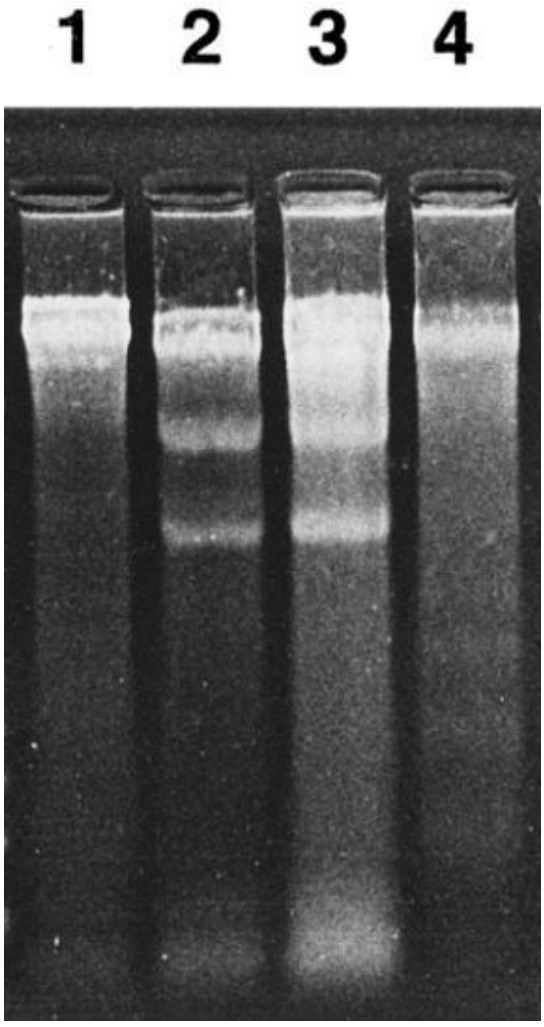



**Image.4.4.1:** DNA fragmentation by agarose gel electrophoresis of *Pandanus odorattissimus* L. Lane 1- 1 kb ladder, Lane2- DNA from cells treated with 25 µg/ml sample, Lane 3- DNA from cells treated with 50 µg sample, Lane 4- untreated DNA.

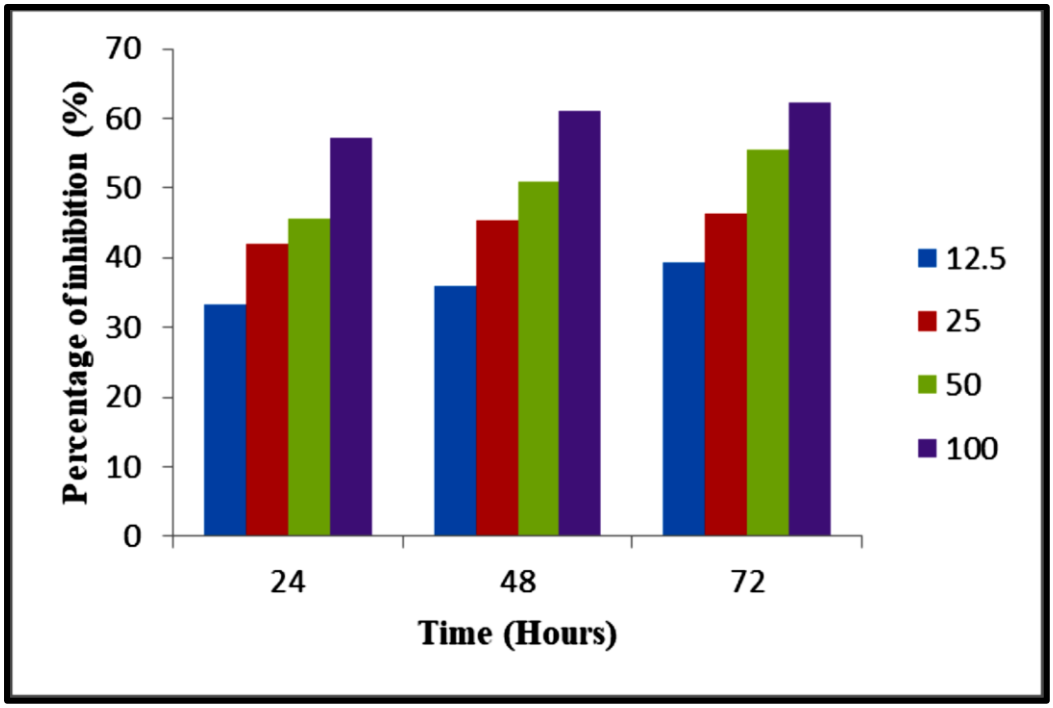



**Figure: 4.4.1.**Cell growth inhibition for *Pandanus odorattissimus* L. methanol extract against KB cell lines at 24 h, 48 h and 72 h.

# Chapter 5

# DISCUSSION

The potency of phenolic compound 4-(4-(3,4-dimethoxyphenyl)hexahydrofuro[3,4-c]furan-1-yl)-2-methoxyphenyl acetate as anticancer agent has been judged by measuring the i) inhibition of cell growth, ii) reduction in tumor weight and iii) increase in mean survival time of the EAC bearing mice [96]. For EAC bearing mice, tumor weight has been found to increase rapidly with time. The treatment of mice with test compound reduced tumor growth rate. Similar trend has been found in cell growth inhibition ability. The lifespan of EAC bearing mice increased remarkably when treated with phenolic compounds. The prolongation of lifespan of cancer bearing mice is a very important and reliable criterion for judging the potency of any drug as anticancer agent [97]. Positive effect of the compound on EAC bearing mice has further been verified by monitoring the change in hematological and biological parameters.

Both the RBC and hemoglobin content of EAC bearing mice were found to be decreased gradually with time, as found in normal mice. This is probably owing to the deficiency of iron in hemolytic or myelopathic condition [98]. Treatment of mice with tested phenolic compound has changed RBC and hemoglobin contents towards normalcy. With the growth of tumor, WBC level increased with time. Rise of WBC count of the treated EAC bearing mice was slower than that of untreated EAC bearing mice. Parallel hematological experiments were done with normal mice to evaluate the host toxic effect of compound. A very slight deterioration in such parameters has been observed during the treatment period. Similar toxic effects were observed in Vinblastine 0.5 mg/kg (i.p.) treated mice [99]. The immunological effect of test compound in fresh healthy mice were performed by counting peritoneal macrophages that have provided further support for potency of the compound as anticancer agent.

Tested phenolic compound notably increased the number of macrophages. This enhancement might produce some cytokinetic products, such as tumor necrosis factor (TNF), interleukins (IL), interferons (IF) etc. which in turn may be responsible in destroying tumor cells [100]. Based on the above results, it can be concluded that, test compound exhibits pronounced activity as an anticancer agent against EAC bearing cells in Swiss albino mice. However, the information presented here is scanty for the said compound to be used as a novel anticancer agent in clinical practice. Many more investigations need to be carried out with this compound and its derivatives using various cancer cell lines with different animal models [101]. Thus, the present study reveals anticancer potential of the tested phenolic compound against EAC in experimental models.

The cytotoxic effect of phenolic compound on HL-60 cells. Data from this study clearly indicates that the phytochemical compound from methanolic extract is highly cytotoxic to human leukemia (HL-60) cells, showing $LD_{50}$ values of 12.24 ± 0.5 µg/mL, 10.25 ± 0.3 µg/mL and 5.2 ± 0.6 µg/mL for 12, 24 and 48 hours of exposure, respectively. Recently, it has been observed that the compound is cytotoxic to human liver carcinoma (HepG2) cells, showing $LD_{50}$ of 8.55 ± 0.58 µg/ml after 48 hours of exposure [102]. We found that low dose of the compound has induced minimal toxicity in HL-60 cells upon 48 hours of exposure. Interestingly, such doses are similar to the therapeutically effective concentrations of compound which have been shown to induce remission in APL patients with minimal toxicity. Clinically, the standard dose for the treatment of patients with APL is 0.15 mg/kg per day which yields a maximum dose of 2-3 µM of

compound in the plasma. High levels of the compound (5 µg/mL and higher for 48 hrs) induce more than 50% of cell mortality [103].

The results indicate that high levels of phenolic compounds exposure of HL-60 cells has inhibited cell proliferation and induced mortality in a dose and time - dependent manner. Such effects have been observed with other test models, as well as clinically studied [104,105]. Current study proves the potentiality of isolated compound as anticancerous agent.

Apoptosis is an important homeostatic mechanism that balances, cell division and cell death, thus maintaining the appropriate cell number in the body [106]. Disturbances of apoptosis in cancer cells have been studied in detail, and induction of apoptosis was one of the strategies for anticancer drug development [107,108]. Emerging evidence has demonstrated that the anticancer activities of certain chemotherapeutic agents are involved in the induction of apoptosis and have no side effects on normal tissues, which are regarded as the preferred method of treating cancer [109]. Apoptosis is a form of programmed cell death that is characterized by a variety of morphological features, including changes in the plasma membrane such as loss of membrane asymmetry and attachment, cell shrinkage, chromatin condensation and chromosomal DNA fragmentation [110].

The findings of this study reveals that phenolic compound isolated from *Pandanus odorattissimus* L. compound has efficient apoptotic activity on HL-60 cells and the number of apoptotic cells increased significantly after treatments. Data obtained from cell morphological assessment showed that *Pandanus odorattissimus* L. compound could induce HL-60 cell

shrinkage as well as nuclear fragmentation marginalization, chromatin condensation and production of membrane-bound apoptotic bodies. These results indicated that *Pandanus odorattissimus* L. compound may induce apoptosis in HL-60 cells.

We have examined the cytotoxic effect of phenolic compound on MDA-MB-231 cells. Data from this study clearly indicates that the phytochemical compound isolated from methanolic extract is highly cytotoxic to MDA-MB-231 cells, showing $LD_{50}$ values of 12.24 ± 0.5 μg/mL, 10.25 ± 0.3 μg/mL and 5.2 ± 0.6 μg/mL for 24, 48 and 72 hours of exposure, respectively. Recently, it has been observed that the compound is cytotoxic to human liver carcinoma (HepG2) cells, showing an $LD_{50}$ of 8.55 ± 0.58 μg/ml after 72 hours of exposure [111]. We found that low dose of the compound has induced minimal toxicity in MDA-MB-231 cells upon 72 hours of exposure. Interestingly, such doses are similar to the therapeutically effective concentrations of compound which have been shown to induce remission in APL patients with minimal toxicity. Clinically, the standard dose for the treatment of patients with APL is 0.15 mg/kg per day which yields a maximum dose of 2-3 μM of compound in the plasma. High levels of the compound (5 μg/mL and higher for 72 hrs) induce more than 50% of cell mortality [112].

In the present study, the results indicate that high levels of phenolic compounds exposure to MDA-MB-231 cells has inhibited cell proliferation and induced mortality in a dose and time - dependent manner. Such effects have been observed with other test models, as well as clinically studied [113,114]. Current study proves the potentiality of isolated compound as anticancerous agent.

Medicinal plants constituents a common alternative for prevention of cancer and treatment in many countries around the world [115]. In the present study, it was shown that phenolic compounds inhibit cancer cells by metabolizing enzymes that alter metabolic activation of potential carcinogens. The mechanism of action of anti-cancer activity of phenols is by disturbing the cellular division during mitosis at the telophase stage. It has also been reported that phenols reduce the amount of cellular protein and mitotic index and colony formation during cell proliferation of cancer cells [116]. So they are regarded as promising anticancer agents against most human cancers [117,118]. MTT assay is a universally accepted *in vitro* method for screening the drugs having cytotoxic activity. *In vitro* cytotoxic activity against MDB-MB-468 cell line at different concentration of phenolic compound of *Pandanus odorattissimus* L. was evaluated in the present study. Phenolic compound inhibited the growth of MDB-MB-468 breast cancer cell lines and had strong cytotoxicity in a concentration-dependent manner. Cytotoxic effect against the MDB-MB-468 cell line is considered as a prognostic anticancer activity. $IC_{50}$ value of phenolic compound was found to be20 μg/ml.

The normal fibroblast was used as a cell is very difficult to culture. Therefore, the present study was designed to test only extracts that are known to show high toxicity with breast cancer cell lines and L929 was used as a representative of a normal cell. Mouse fibroblast L929 is a normal cell line, which is recommended by international standards for testing medical devices [119] and responds more sensitively than primary cells [120]. A plant extract that will act successfuly as an anti-cancer drug should kill cancer cells without causing excessive damage to

normal cells, such as L929, while MDA-MB-468 cells cause p53 mutations [121]. Furthermore, MDA-MB-468 cells is ER negative and classified as high-grade and basal type [122]. Our results showed that 20 μg/ml had more cytotoxic effects on MDA-MB-468, a high-grade cancer. Surprisingly, it appeared less toxic in normal fibroblast cells than breast cell lines. These results suggest that all fractions were more selective for breast cells than normal cells.

This phenomenon can be generally detected by agarose gel electrophoresis. Therefore, DNA ladder formation indicated that the cytotoxic effect of 20μg/ml caused inhibition in the growth of breast cancer and normal fibroblast cells through apoptosis. 20 μg/ml also inhibited growth in normal breast cells, but not to the point of death through apoptosis [123]. In the L929 cells, 20 μg/ml inhibited cell growth within four days and induced cell death on Day 5 after incubation. Although treated L929 cells showed DNA ladders at Day 5, the incubation time was longer and the dose was higher than in the breast cancer cell lines. Thus 20μg/ml seemed to be safe for normal cells.

Apoptosis is a programmed cell death that specifically removes or eliminates unwanted or dead cells. Unlike shrunken cells, the characteristics of apoptotic cells include condensation of the cytoplasm and nucleus, aggregation of chromatin and formation of membrane-bound vesicles called apoptotic bodies [124]. In contrast, necrosis denotes a pathological activity. Necrosis is known to be pro inflammatory and is characterized by cell swelling that is often accompanied by chromatin condensation. Necrotic cells eventually developed cellular and nuclear lysis followed by inflammation [125],which would be disadvantageous for an anticancer agent. Many plant

derived cancer drugs have been clinically useful;include vinblastine, irinotecan, topotecan and paclitaxel [126,127].

Various biological processes and biological interactions occur between flavonoids, phenolic compounds or polyphenols, with enzymes, proteins that make them toxic to the cell or inhibit the growth of cells [128].The conventional anticancer drugs that are used act on both normal cells and tumor cell and cause brutal side effects and tumor resistance. Anticancer activities through apoptotic induction by herbs show no side effects [129]. Natural herbs can be used extensively to prevent and treat cancer. Caspases are responsible for the programmed cell death or apoptosis. It starts with the activation of intrinsic and extrinsic paths. Once activated, these destructive proteases systematically break down the cell to ensure its effective removal without damage to surrounding cells and tissues. Hence *Pandanus odorattissmus* L. helps by producing these caspases to induce apoptosis in the oral cancer cell line.

The current study showed that the presence of phytochemicals in selected plant leaves extract has an effective cytotoxic activity against oral cancer cells. The exact compounds responsible for the plant's anticancer activity will help in the search of new anticancer agents.

# CONCLUSION

The phenolic compound 4-(4-(3,4-dimethoxyphenyl)hexahydrofuro[3,4-c]furan-1-yl)-2-methoxyphenyl acetate has been checked for the cell viability in duration of 12, 24 and 48 hours, in which the 48 hrs duration has shown a significant inhibition of cell viability. Thus the inhibition of cancer after the treatment of pure extract of phenolic compound on the HL-60 cell line indicates that the compound is a potent drug for the cancer treatment. The phenolic compounds exposure to HL-60 cells has inhibited cell proliferation and induced mortality in a dose and time - dependent manner. The phenolic compound has efficient apoptotic activity on HL-60 cells and the number of apoptotic cells increased significantly after treatments.

The phenolic compound when checked for the cell viability in duration of 24, 48 and 72 hours the duration of 48 hrs duration has shown a significant inhibition of cell viability. Thus the inhibition of cancer after the treatment of pure extract of phenolic compounds on the MDA-MB-231 cell line indicates that the compound is a potent drug for the cancer treatment. The phenolic compounds exposure of MDA-MB-231 cells has inhibited cell proliferation and induced mortality in a dose and time - dependent manner.

The medicinal plant constitutes a common alternative for cancer prevention and treatment in many countries around the world. The compound has been isolated from *Pandanus odorattissimus* L. which is a endangerous plant species is used for the treatment of various human ailments for many years. In the present study, the results of anticancer activity of compound showed a potent cytotoxic activity against MDB-MB-468 breast cancer cell lines. Thus the inhibition of cancer after the treatment of phenolic compounds 4-(4-(3,4-

dimethoxyphenyl) hexahydrofuro[3,4-c]furan-1-yl)-2-methoxyphenyl acetate on the MDB-MB-468 cell line indicates that the compound is a potent drug for the cancer treatment.

The mechanism that can represent a possibly viable approach for effective tumor treatment that has several advantages over conventional therapies and the more current "designer" approaches.

The secondary metabolites of herbs are always promising with anticancer activity. The ability of apoptotic induction by *Pandanus odorattissmus* L. extract can be used in anticancer formulation. The cancer inhibition effects of certain medicinal herbs can therefore be used to treat trauma over a longer period of time, which is always very promising. Therefore, *Pandanus odorattissmus* L. can be used in the treatment of cancer.

# REFERENCE

1. Liu, T.; Men, Q.; Wu, G.; Yu, C.; Huang, Z.; Liu, X.; Li, W. Tetrandrine induces autophagy and differentiation by activating ROS and Notch1 signaling in leukemia cells. Oncotarget 2015, 6, 7992–8006. [CrossRef] [PubMed]

2. Wong, V.K.; Chiu, P.; Chung, S.S.; Chow, L.M.; Zhao, Y.Z.; Yang, B.B.; Ko, B.C. Pseudolaric acid B, a novel microtubule-destabilizing agent that circumvents multidrug resistance phenotype and exhibits antitumor activity in vivo. Clin. Cancer Res. **2005**, 11, 6002–6011. [CrossRef] [PubMed]

3. Balachandran, P.; Govindarajan, R. Cancer—Anayurvedic perspective. Pharmacol. Res. 2005, 51, 19–30. CrossRef] [PubMed].

4. *G. Babu&Jeenachandran. Int. Res. J. Pharm. 2019, 10 (1).*

5. Samkeliso Takaidza1, Arumugam Madan Kumar2, Cornelius Cano Ssemakalu1, NagabishekSirpu Natesh2, Gayathri Karanam2, Michael Pillay1. Anticancer activity of crude acetone and water extracts of Tulbaghiaviolaceaon human oral cancer cells. Asian Pacific Journal of Tropical Biomedicine 2018; 8(9): 456-462.

6. Mbele M, Hull R, Dlamini Z. African medicinal plants and their derivatives: Current efforts towards potential anti-cancer drugs. ExpMolPathol2017; 103(2): 121-134.
7. Teiten MH, Gaascht F, Dicato M, Diederich M. Anticancer bioactivity of compounds from medicinal plants used in European medieval traditions. BiochemPharmacol2013; 86(9): 1239-1247…

8. Chung DM, Kim JH, Kim JK. Evaluation of MTT and Trypan Blue assays for radiation-induced cell viability test in HepG2 cells. IntJRadiat Res 2015; 6(6): 713-724.

9. Formigli L, Papucci L, Tani A, Schiavone N, Tempestini A, Orlandini G, *et al.*, Aponecrosis: Morphological and biochemical exploration of a syncretic process of cell death sharing apoptosis and necrosis. J Cell Physiol2000; 182(1): 41-49.

10. Damodaran B, Nagaraja P, Jain V, ManujaVirajWimalasiri MP, Sankolli GM, Kumar GV, *et al.*, Phytochemical screening and evaluation of cytotoxic activity of *Calotropisgiganteal*eaf extract on MCF7, HeLa, and A549 cancer cell lines. J Nat ScBiol Med 2019;10:131-8.

11. UrszulaGawlik-Dziki , MichałS´wieca , MaciejSułkowski , DariuszDziki , Barbara Baraniak , JarosławCzyz_. Antioxidant and anticancer activities of Chenopodiumquinoa leaves extracts – In vitro study .foof and chemical toxicology. (2013) 57, 154-160.

12. W.D. Wan Chik, A. Amid and P. Jamal, 2010. Purification and Cytotoxicity Assay of Tomato (*Lycopersiconesculen tum*) Leaves Methanol Extract as Potential Anticancer Agent. *Journal of Applied Sciences, 10: 3283-3288.*

13. Samantha L. Gerlach, Ramesh Rathinakumar, Geetika Chakravarty, Ulf Göransson, William C. Wimley, Steven P. Darwin , DebasisMondal,.Anticancer and chemosensitizing abilities of cycloviolacin O2 from *Viola odorata* and psylecyclotides from *Psychotrialeptothyrsa.* Peptide science-2010, 617- 625.

14. Raju AB, Ravindranath A, Kalpana G. Antitumor activity of *Diospyrosperegrina* on Ehrlich ascites carcinoma in mice. J Sci Res. 2011;3:413–19.

15. J. M. Brown and L. D. Attardi, "The role of apoptosis in cancer development and treatment response," *Nature Reviews Cancer*, vol. 5, no. 3, pp. 231–237, 2005.View at: Publisher Site | Google Scholar.

16. Narendhran, S., Rajiv, P., Vanathi, P., and Sivaraj, R. (2014). Spectroscopic analysis of bioactive compounds from *Streptomyces cavouresis* KUV39: evaluation of antioxidant and cytotoxicity activity. *Int. J. Pharm. Pharm. Sci.* 6, 319–322.

17. Overall CM and López-Otín C: Strategies for MMP inhibition in cancer: innovations for the post-trial era. Nat Rev Cancer. 2:657–672. 2002.

18. Mitscher, L.A.; Telikepalli, H.; McGhee, E.; Shankel, D.M. Natural antimutagenic agents. *Mutat. Res.* **1996**, *350*, 142–143.

19. Owen, R.W.; Giacosa, A.; Hull, W.E.; Haubner, R.; Spiegelhalder, B.; Bartsch, H. The antioxidant/anticancer potential of phenolic compounds isolated from olive oil. *Eur. J. Cancer* **2000**, *36*, 1235–1247.

20. Sala, A.; Recio, M.; Giner, R.M.; Manez, S.; Tournier, H.; Schinella, G.; Rios, J.L. Anti-inflammatory and antioxidant properties of *Helichrysumitalicum*. *J. Pharm. Pharmacol.* **2002**, *54*, 365–371.

21. Kenny, P.A.; Lee, G.Y.; Myers, C.A.; Neve, R.M.; Semeiks, J.R.; Spellman, P.T.; Lorenz, K.; Lee, E.H.; Barcellos-Hoff,M.H.; Petersen, O.W.; *et al.*, The morphologies of breast cancer cell lines in three-dimensional assays correlate with their profiles of gene expression. *Mol. Oncol.* **2007**, *1*, 84–96.

22. Kerr JF, Winterford CM, Harmon BV, Apoptosis. Its significance in cancer and cancer therapy. Cancer. 1994. 73: 2013- 26.

23. A. Mohammadi1,2, B. Mansoori1, 2, S. Goldar1, D. Shanehbandi1, V. khaze1, L. Mohammadnejad1, E. Baghbani1, B. Baradaran1. Effects of Urticadioica dichloromethane extract on cell apoptosis and related gene expression in human breast cancer cell line (MDA-MB-468)*Cell. Mol. Biol.*2016, 62 (2): 62-67.

24. Mohamme. dShafi Sofi1, M.K. Sateesh*1 and Mohsin Bashir. Screening of the Ethnobotanicals against MDA-MB-231 and MCF-7 breast cancer cell lines International Journal of Phytopharmacy Research Article Vol. 4 (6), pp.140-147, Nov-Dec 2014.

25. Patel, R. and Patel, S.(2011). Cytotoxic activity of methanolic extract of Artocarpusheterophyllus against A549, Hela and MCF-7 cell lines. Journal of Applied Pharmaceutical Science, 1(7):167-171.
26. Ramya, D. and Vedha, H.(2012). Evaluation of in vitro anticancer activity of hydroalcoholic extract of tabernaemontanadivaricata.AsianJournal of Pharmaceutical and Clinical Research,5 (3) :59-61.

27. Available from: https://www.who.int/news-room/fact-sheets/detail/ cancer, 2019. [Last accessed on 2019 Feb 17].

28. Available from: http://cancerindia.org.in/cancer-statistics/, 2019. [Last accessed on 2019 Feb 17].

29. Safarzadeh E, Sandoghchian SS, Baradaran B. Herbal medicine as inducers of apoptosis in cancer treatment. Adv Pharm Bull 2014; 4(suppl 1): 421-427.

30. Tracey AM, Lin Y, Andrew JS, Jane L, Wen GJ. Cancer invasion and metastasis: Molecular and cellular perspective. LandesBiosci2013; 2: 135-168.

31. MacDonald V. Chemotherapy: Managing side effects and safe handling. Can Vet J 2009; 50(6): 665-668.

32. Liu B, Ezeogu L, Zellmer L, Yu B, Xu N, Joshua-Liao D. Protecting the normal in order to better kill the cancer. Cancer Med 2015; 4(9): 1394- 1403.

33. Gerl R, Vaux DL. Apoptosis in the development and treatment of cancer. Carcinogenesis 2005; 26(2): 263-270.

34. Azmi AS. Adopting network pharmacology for cancer drug discovery. Curr Drug DiscovTechnol2013; 10(2): 95-105.

35. Radha R, Kavimani S, Ravichandran V. Antitumour activity of methanolic extract of PlumeriaalbaL. leaves against Dalton lymphoma ascities in mice. Int J Health Sci Res 2008; 1(2): 79-85.

36. Veeresham C. Natural products derived from plants as a source of drugs. J Adv Pharm Technol Res 2012; 3(4): 200-201.

37. Walter TM, Merish S, Tamizhamuthu M. Review of Alternantherasessiliswith reference to traditional siddha medicine. Int J PharmacognPhytochem Res 2014; 6: 249-254.

38. Chanchal, N. R., and Balasubramaniam, A. (2011), Pharmacogostic and antimicrobial studies of the leaves of *Tabernaemontanadivaricata*R.br.,Pharmacologyonline, 2, pp 1171-1177.

39. Joy, P. P., Thomas J, Samuel Mathew, Baby P Skaria (1998), "Medicinal plants", Kerala Agricultural University, Kerala, India, pp 1-9.

40. Kapadia, G. J., Azuine MA, Tokuda H (2002), Inhibitory effect of herbal remedies on 12-O-tetradecanoylphorbol-13-acetate-promoted Epstein Barr virus early antigen activation, Pharmacology Research,45, 3, pp 213-220., doi:10.1006/phrs.2001.0936.

41. Gupta, M. UpalKantiMazumdar, RamanathanSambathkumar, Thangavel Siva kumar (2004), Antitumor activity and antioxidant race of *Bauhinia racemosa*against EAC in Swiss albino mice, ActaPharamcologiciaSinica, 25, 8, pp 1070-1076.

42. Ho, Y. W., Theodohov, M., Brooks A E., Abdulla, N., Jalaluddin S (1994), Fermentability of some tropical feedstuffs for ruminants as determined by the pressure transducer gas production and the environment. 7th AAAP Animal Science Congress, Bali, Indonesia, Vol 3, 11-16 July, pp 63-64.

43. Abdulaev, F. I., (2001), “Plant derived agent against cancer” Gupta SK, editor, Pharmacology and Therapeutics in the new millennium, Narosa publishing house, New Delhi, pp 345-54.

44. Belinsky, M.,and Jaiswal, A. K., (1993), NAD(P)H: Quinone oxidoreductase 1 (DT-diaphorase) expression in normal and tumor tissue, Cancer Metastasis Reviev, 12, 2, pp 103-117.

45. Hakama, M., (1997), Chemoprevention research in Europe, International Journal of Cancer, 71, S10, 30-33.,

46. Monks NR, Bordignon SAL, Ferraz A, Machado KR,Faria DH, Lopes RM, Mondin CA, De Souza ICC, Lima MFS, da Rocha AB, Schwartsmann G. 2002. Anticancer screening of Brazilian plant. *Pharmaceut. Biol.* 40(8): 603-616.

47. Cai Y, Lou Q, Sun M, Coorke H. 2004. Antioxidant activity and phenolic compounds of 112 traditional Chinese medicinal plants associated with cancer. *Life Science*, 74: 2157-2184.

48. Zahra Amirghofram, MasoudBahmani, Abbas Azadmehr, KatayounJavidnia. 2006. Induction of apoptosis in leukemia cell lines by Linumpersicum and Euphorbia cheiradenia. *Journal of Res ClinOncol.* 132: 427-432.

49. Grune& Stratton Inc. 1987. Fried, Edrita Publisher: New York.

50. D. Morgan, F. Russetti, R. Gallo. 1976. Selective in vitro growth of T lymphocytes from normal human bone marrows. *Science* 193, 1007.

51. Shahedur R, Faizus S, Asif I, BMC Complementary and Alternative Medicine, 11, 76, 2011.

52. Weimin Z, Lili Z, Jürgen R, Pharm Biol, 9, 886, 2009.

53. Dongwu L, Zhiwei C, J Breast Cancer, 2, 133, 2013.

54. Schwartsmann G, Ratain MJ, Cragg GM, Wong JE, Saijo N, Parkinson DR *et al.*, Anticancer drug discovery and development throughout the world. Journal of Clinical Oncology;20:47S–59S, 2002.

55. Newman DJ, Cragg GM, SnaderKM.Natural products as sources of new drugs over the period. J. Nat. Prod;66:1022-37, Jul 2003.

56. Srivastava SK, Rai V, Srivastava M, Ra AK, wat S, Mehrotra S. Estimation of heavy metals in different Berberis Species and its market samples. Environ Monit Assess.;116:315-20, May 2006.

57. Gupta SC, Kim JHS, Prasad BB. Regulation of survival, proliferation, invasion, angiogenesis, and metastasis of tumor cells through modulation of inflammatory pathways by nutraceuticals, Cancer Metastasis Rev;29: 405, Sep 2010.

58. Sun Y, Xun K, Wang Y, Chen X. A systematic review of the anticancer properties of berberine, a natural product from Chinese herbs. Anticancer Drugs;20:757-69, 2009.

59. Siegel R, Ma J, Zou Z, Jemal A (2014). A Cancer Statistics. CA Cancer J. Clin. 64(1): 9-29.

60. Cancer Research UK (2013). Side Effects of Cancer Drugs. Available online: http://cancerhelp.cancerresearchuk.org/about-cancer/treatment/cancer-drugs/side-effects.

61. Monks NR, Bordignon SAL, Ferraz A, Machado KR, Faria DH, Lopes RM, Mondin CA, De Souza ICC, Lima MFS, da Rocha AB, Schwartsmann G (2002). Anticancer screening of Brazilian plant. Pharm. Biol. 40(8): 603-616.

62. Cai Y, Lou Q, Sun M, Coorke H (2004). Antioxidant activity and phenolic compounds of 112 traditional Chinese medicinal plants associated with cancer. Life Sci. 74(17): 2157-2184.

63. Kummalue T, O-charoenrat P, Jiratchariyakul W (2007). "Antiproliferative effect of *Erycibeelliptilimba*on human breast cancer cell lines." J. Ethnopharmacol. 110(3): 439-443.

64. Engel JB, Schally AV, Halmos G, Baker B, Nagy A, Keller G (2005). Targeted cytotoxic bombesin analog AN-215 effectively inhibits experimental human breast cancers with a low induction of multi-drug resistance proteins. Endocr. Relat. Cancer. 12(4): 999-1009.

65. Cailleau R, Olive M, Cruciger QVJ (1978). Long-term human breast carcinoma cell lines of metastatic origin: preliminary characterization. *In Vitro*. 14(11): 911-915.

66. Gordon MC, David JN (2005). Plants as a source of anti-cancer agents. J. Ethnopharmacol. 100(1-2): 72-79

67. Lee KH (1993). Human medicinal agents from plants. In: Kinghorn AD, Balandrin M, editors. ACS Symposium Series No. 534.Washington, DC: American Chemical Society. Chap.12.

68. Nataru S, Pulicherla Y, Gaddala B. A Review on medicinal plants as a potential source for cancer. Int J Pharm Sc Rev Res. 2014;26(1):235-48.

69. Huang L, Feng ZL, Wang YT, Lin LG. Anticancer carbazole alkaloids and coumarins from Clausena plants: A review. Chin J Nat Med. 2017;15(12):881-8.

70. Sewram V, Sitas F, O'connell D, Myers J. Tobacco and alcohol as risk factors for oesophageal cancer in a high incidence area in South Africa. Cancer Epidemiol. 2016;41:113-21.

71. Kumar M, Nanavati R, Modi T, Dobariya C. Oral cancer: Etiology and risk factors: A review. J Cancer Res Ther. 2016;12(2):458-63.

72. Herrero RCX, Pawlita M, Lissowska J, Kee F, Balaram P, Rajkumar T, *et al.*, Human papillomavirus and oral cancer: The International Agency for Research on cancer multicenter study. J Natl Cancer Inst. 2003;95(23):1772-83.

73. Huang FL, Yu SJ. Esophageal cancer: Risk factors, genetic association, and treatment. Asian J Surg. 2018;41(3):210-5.

74. Uddin Q, Siddiqui KM, Rais-Ur-Rahman. Unani Medicine for Cancer Care: An evidence-based review. Int J Ayu Herb Med. 2015;5(3):1811-25.

75. Kabary HA, Attia M, Easa SM, Awad NM, Abou-Shanab RAI, Eida MF. Res J Pharm, BiolChem Sci. 2012.2.

76. NAPRALERT, 1993. Biological activities for extracts of *Pandanus* and ethnomedical information on *Pandanus*. University of Illinois at Chicago. Chicago,IL.

77. Wilson. K and Walker. J. (1995). Practical Biochemistry; Principles and techniques, 4th Edn. Published by Cambridge University Press, Cambridge, UK. Pp 586

78. Ting ting Jong and Shang whang chau. (1998). Antioxidative activities of constituents isolated from Pandanus odoratissimus. *Phytochemistry*. 49(7)**:** 2145-2148.

79. Wing-Yan, Li., Shun-Wan, C.,De-Jian, Guo, and Peter, Hoi-Fu Yu.(2007).Correlation Between antioxidative power and anticancer Activity in Herbs from Traditional Chinese Medicine Formulae with Anticancer Therapeutic Effect. Pharmaceutical Biology, 45(7):541-546.

80. Y. Rajeshwar, Manali Gupta, Kishor Mazumder. Antitumor activity and in vivo antioxidant status of Mucuna pruriens (Fabaceae) seeds against Ehrlich ascites carcinoma in Swiss albino mice**.** Iranian Journal of Pharmacology and Therapeutics 4(1):46-53 · January 2005

81. B Rajkapoor , B Jayakar , N Murugesh Antitumor activity of Indigofera aspalathoides on Ehrlich ascites carcinoma in mice. Indian journal of pharmacology. 2004. vol 34, (1). 38-40

82. Amritpal Singh, Sanjeev Duggal1 , Harmanpreet Singh1 , Jaswinder Singh2 , Shankar Katekhaye Withanolides: Phytoconstituents with significant pharmacological activities..International Journal of Green Pharmacy 2010, 229 -237.

83. S. Gayatri, C. Uma Maheswara Reddy, K. Chitra, and V. Parthasarathy. Assessment of *in vitro* cytotoxicity and *in vivo* antitumor activity of *Sphaeranthus amaranthoides* burm.f. Pharmacognosy Res. 2015 Apr-Jun; 7(2): 198–202.

84. Ganesh Chandra Jagetia, Ponemone Venkatesh, Manjeshwar Shrinath Baliga. Aegle marmelos (L.) Correa inhibits the proliferation of transplanted Ehrlich ascites carcinoma in mice**.** Biol Pharm Bull. 2005 Jan;28(1):58-64.

85. Senthilkumar Natesan, Shrishailappa Badami, Santoshkumar H Dongre, Ashok Godavarthi

86. Antitumor activity and antioxidant status of the methanol extract of Careya arborea bark against Dalton's lymphoma ascites-induced ascitic and solid tumor in mice**.** J Pharmacol Sci. 2007 Jan;103(1):12-23.

87. Linda S M Ooi [1], Hua Wang, Choi-Wan Luk, Vincent E C Ooi. Anticancer and antiviral activities of Youngia japonica (L.) DC (Asteraceae, Compositae)**.**J Ethnopharmacol . 2004 Sep;94(1):117-22.

88. Min-Jung Kim, Do-Hee Kim, Hye-Kyung Na, Tae Young Oh. Eupatilin, a Pharmacologically Active Flavone Derived from Artemisia Plants, Induces Apoptosis in Human Gastric Cancer (AGS) Cells.Journal of Environmental Pathology Toxicology and Oncology 24(4):261-9

89. Duangjai Tungmunnithum, Areeya Thongboonyou,Apinan Pholboon and Aujana Yangsabai. Flavonoids and Other Phenolic Compounds from Medicinal Plants for Pharmaceutical and Medical Aspects: An Overview. Medicines (Basel). 2018 Sep; 5(3): 93.

90. Yamaguchi, K. (1970). Spectral data of Natural Products Vol. I Elsevier Publishing Company, Amsterdam, London, New York..

91. Keerthikar and Basu: (2000) *Indian Medicinal Plant, Sri Satguru Publication*. Vol. **10** p. 3565.

92. Warrier R.k., V.P.K. Nambiar and C.Ramankutty, 1995. Indian Medicinal Plants: a compendium of 500 species: Orient Longman Ltd. Madras. India

93. Jesmin, M., Ali MM, Khanam JA (2010), Antitumor activities of some Schiff bases derived from benzoin, salicylaldehyde, amino phenol and 2, 4-dinitrophenyl hydrozine. Thai Journal of Pharmaceutical Science, 34, 1, 20-31.

94. Hoagland, H. C., (1982), Hematological complication of cancer chemotherapy. Seminars in Oncology, 9, 1, pp 95-102.

95. Hudson, L., and Hay, F. C., (1989), “Isolation of normal peritoneal macrophages”. In Practical

96. Immunology, 3rd edn, L. Hudson, F.C. Hay (eds) Blackwell Sci. Pub. Oxford, London, pp 26-28.

97. Chandran, A., and Arunachalam, G., (2015), Evaluation of in vivo anticancer activity of *Scaevola taccadea* Roxb against Ehrlich Ascites Carcinoma in Swiss Albino Mice. Journal of Pharmaceutical Science and Research, 7, 9, pp 626-632.

98. Clarkson, B. D., and Buirchenal, J. H., (1965), Preliminary screening of antineoplastic drugs. Progress in Clinical Cancer, 10, pp 625-663.

99. Fenninger, L. D., and Mider, G.B., (1954), Energy and nitrogen metabolism in cancer. Advances in Cancer Research, 2, pp 229-53.

100. Burger, A., (1981), “Medicinal Chemistry”, Vol 2 (3rd ed.), John Wiley and Sons, London, pp 602-53.

101. Lee, N. N., Cadman EC, Michael IN (1982), Randomized study comparing doxorubicin, cyclophosphomide, vincristine, methotrexate with leucovorin rescue and cytarabine (ACOMLA) with cyclophosphamide, doxorubicin, vincristine, prednisone and bleomycin (CHOP-B) in the treatment of diffuse histiocytic lymphoma. Cancer Treatment Report, 66, 6, pp 1279-1284.

102. Islam, K., Sheikh M Mohsin Ali, Mele Jesmin, Jahan Ara Khanam (2012), *In vivo* anticancer activities of benzophenone semicarbazone against Ehrlich Ascites Carcinoma

Cells in Swiss Albino mice. Cancer Biology and Medicine, 9, 4, pp 242-247., doi: 10.7497/j.issn.2095-3941.2012.04.004.

103. J. Paul. 1975. Cell and Tissue Culture. Churchill Livingstone (CSL).

104. Mosmann. T. 1983. Rapid colorimeteric assay for cellular growth and survival: applications to proliferation and cytotoxicity assays. *J Immuno Methods* 65(1-2) 55-63.

105. Son Yo, Kim JC, Chung GH, Lee JC. 2003. Ripe fruits of Solamin nigrum L inhibits cell growth and induces apoptosis in MCF-7 cells. *Food Chemi. Toxicol* 41: 1421-1428.

106. Collins AR, Oscoz AA, Brunborg G, Gaivao I, Giovannelli L, Kruszsewki M, Smith CC, Stetina R. 2008. The comet assay: the topical issue. *Mutagenesis* 17: 1-9.

107. Singh NP, McCoy MT, Tice TT, Schneider EL. 1988. A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp. Cell Res*, 175: 184-191.

108. Hartmann A, Agurelli E, Beevers C, Brendler SS, Burlinson B, Clay P, Collins A, Smith A, Speit G, Thybaud V, Tice RR. 2003. Recommendation for conducting the in vivo alkaline comet assay. *Mutagenesis* 18: 45-51.

109. Grusch M, Pollgar D, Gfatter S, Leuhuber K, Huettenbrenner S, Leisser C, Fuhrmann G, Steinkellner H, Smid K, Peter GJ, Jayaram HN, Klopal W, Szekeres T, Knasmuller S, Krupitza G. 2002. Maintenance of ATP favours apoptosis over necrosis triggered by benzamide riboside. *Cell Death Difference* 9(2): 169-178.

110. P. B. Tchounwou, C. G. Yedjou, W. C. Dorsey. 2003. Arsenic trioxide induced transcriptional activation and expression of stress gene in human liver carcinoma cells (HepG2). *Cellular and molecular biology* 49(7): 1071- 1079.

111. S. L. Soignet, S. R Frankel, D. Douer, M. S. Tallman, H. Kantarjian, E. Calleja, R. M.

112. Stone, M. Kalaycio, D. A. Scheinberg, P. Steinherz, E. L. Sievers, S. Coutre, S. Dahlberg, R. Ellison, R. P. Warell. 2001. United States multicenter study of arsenic trioxide in relapsed acute promyelocytic leukemia. *J. Clin. Oncol.* 19: 3852-3860..

113. P. Fenaux, C. Chomienne, L. Degos. 2001. Treatment of acute promyelocytic leukaemia. *Clin Haematol.* 14: 153-174.

114. J. Mayorga, C. Richarson-Hardin, K.A. Dicke. 2002. Arsenic trioxide as effective therapy for relapsed acute promyelocytic leukemia. *Clin J Oncol Nurs.* 6(6): 341-346.

115. Ozelm Sultan Aslantutk and Tulay Abkin Cel K. 2013. Antioxidant, cytotoxic, and apoptotic activities of extracts of medicinal plant euphorbia platyphyllos L. *Journal Of Medicinal Plants Research* 7(19): 1293-1304..

116. Martin SJ, Green DR. 1995. Apoptosis and cancer the failure of controls on cell death and cell survival. *Criti Rev Oncol. Hematol.* 18: 137-153.

117. Hu W, Kanavagh JJ. 2003. Anticancer therapy targeting the apoptitic pathway, *Lancet Oncol.* 4: 721-729.

118. Xiao JX, Huang GQ, Zhang SH. 2007. Soya saponins inhibit the proliferation of HeLa cells by inducing apoptosis. *Exp. Toxicol. Pathol.* 59: 35-42.

119. Finkel E. 1999. Does cancer therapy trigger cell suicide.*Science* 289: 2256-2258.

120. Oberhammer F, Wilson JW, Dive C, Morris ID, Hickman JA, Wakeling AE, Walker PR, Sikorska M (1993). Apoptotic death in epithelial cells: cleavage of DNA to 300 and/or 50 kb fragments prior to or in the absence of internucleosomal fragmentation. EMBO J. 12(9): 3679-3684.

121. Paul J (1975). Cell and Tissue Culture. Churchill Livingstone (CSL). Potchanapond G, Mananya M, Yaowapa S (2015). Cytotoxic Activity of *Piper cubeba* Extract in Breast Cancer Cell Lines. Nutrients. 7(4): 2707-2719

122. Schedle A, Samorapoompichit P; Rausch-Fan XH, Franz A, Fureder W, Sperr WR, Sperr W, Ellinger A, Slavicek R, Boltz-Nitulescu G (1995). Response of L-929 fibroblasts, human gingival fibroblasts, and human tissue mast cells to various metal cations. J. Dent. Res. 74(8): 1513-1520.

123. Siegel R, Ma J, Zou Z, Jemal A (2014). A Cancer Statistics. CA Cancer J. Clin. 64(1): 9-29.

124. Soobratte MA, Bahorun T, Aruoma OI (2006). Chemopreventive actions of polyphenolic compounds in cancer. Biofactors. 27(1-4): 19-35.

125. Tan W, Lu J, Huang M, Li Y, Chen M, Wu G (2011). Anti-cancer natural products isolated from chinese medicinal herbs. Chin. Med. 6(1): 27.

126. Umadevi M, Sampath KKP, Debjit B, Duraivel S (2013). Traditionally used anticancer herbs in India. J. Med. Plants Stud. 1(3): 56-74

127. Cohen JJ. Apoptosis. Immunology Today. 1993;14(3):126-30.

128. Wyllie AH, Kerr JF, Currie AR. Cell death: The significance of apoptosis. Int Rev Cytol. 1980;68:251-306.

129. Teiten MH, Gaascht F, Dicato M, Diederich M. Anticancer bioactivity of compounds from medicinal plants used in European medieval traditions. Biochem Pharmacol. 2013;86(9):1239-47.